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BEFORE THE BOARD OF PATENT APPEALS AND INTERFERENCES

Application Number: 09/974,546
Filing Date: October 10, 2001
Appellant(s): AN ET AL.

MAILED

APR 12 2007

GROUP 1600

Tamara A. Kale
For Appellant

EXAMINER'S ANSWER

This is in response to the appeal brief filed January 5, 2007, appealing from the Office action mailed November 10, 2005.

(1) Real Party in Interest

A statement identifying by name the real party in interest is contained in the brief.

(2) Related Appeals and Interferences

The examiner is not aware of any related appeals, interferences, or judicial proceedings which will directly affect or be directly affected by or have a bearing on the Board's decision in the pending appeal.

(3) Status of Claims

The statement of the status of claims contained in the brief is correct.

(4) Status of Amendments After Final

No amendment after final has been filed.

(5) Summary of Claimed Subject Matter

The summary of claimed subject matter contained in the brief is correct.

(6) Grounds of Rejection to be Reviewed on Appeal

The appellant's statement of the grounds of rejection to be reviewed on appeal is substantially correct. The changes are as follows:

WITHDRAWN REJECTIONS

The following ground of rejection is not presented for review on appeal and has been withdrawn by the examiner.

Claim Rejections – 35 U.S.C. § 112

Claims 78-94 were rejected under 35 U.S.C. 112, second paragraph, as being indefinite for failing to particularly point out and distinctly claim the subject matter which applicant regards as the invention.

It had been submitted that claims 78-94 are indefinite because claims 78 and 87 recite the phrase "effective amount". The phrase "effective amount" is indefinite when the claims fail to state the function that is to be achieved. See *In re Frederiksen & Nielsen*, 213 F 2d 547, 102 USPQ 35 (CCPA 1954). As explained, in this instance, it cannot be determined if the claim requires the "effective amount" of said agent to be sufficient to effectively inhibit the peptide or polypeptide, or to effectively treat cancer in the patient, or both. Notably, it is entirely possible that an amount of an agent can effectively inhibit an activity of protein, but be insufficient to inhibit the growth of tumor cells. As such the claims fail to delineate the metes and bounds of the subject matter

that Appellant regards as the invention with the requisite clarity and particularity to permit the skilled artisan to know or determine infringing subject matter.

This ground of rejection has been withdrawn.

GROUNDS OF REJECTION NOT ON REVIEW

The following ground of rejection has not been withdrawn, but is not under review on appeal because it has not been presented for review in the appellant's brief.

Double Patenting

Claims 78-84 and 86-94 are provisionally rejected under the judicially created doctrine of obviousness-type double patenting, as being unpatentable over claims 1-38 and 65-72 of copending Application No. 09/966,762.

Although the conflicting claims are not identical, they are not patentably distinct from each other for the following reasons:

The instant claims are drawn to a method for treating breast, bladder or prostate cancer cells comprising administering an "agent" (e.g., inhibitor), or more particularly an antibody that binds to a peptide or polypeptide encoded by SEQ ID NO: 83 or SEQ ID NO: 85, which the instant specification discloses is a polypeptide comprising the amino acid sequence set forth as SEQ ID NO: 84 and SEQ ID NO: 86.

Claims 1-38 and 65-72 of the copending application are drawn to a method for inhibiting cancer cells in a patient comprising administering to the patient a "UC28 inhibitor", including a polyclonal or monoclonal antibody that binds "UC28".

SEQ ID NO: 84 of the instant application is identical to SEQ ID NO: 2 of the copending application. The protein comprising this amino acid sequence is designated "UC28" by both the instant and copending applications; see, e.g., page 12, lines 24-27 of the copending application; and page 19, line 4, and page 115, lines 11-15 of the instant application.

The claims of the copending application do not explicitly recite that the antibody administered is conjugated or linked to a radionuclide or chemotherapeutic agent; however, the claims of the copending application do explicitly recite that the antibody can be conjugated to a "toxin", which is defined as either a chemotherapeutic agent or a radionuclide (page 89, lines 2 and 3).

This is a provisional obviousness-type double patenting rejection because the conflicting claims have not in fact been patented.

(7) Claims Appendix

The copy of the appealed claims contained in the Appendix to the brief is correct.

(8) Evidence Relied Upon

Stancovski et al. Mechanistic aspects of the opposing effects of monoclonal antibodies to the ERBB2 receptor on tumor growth. *Proc. Natl. Acad. Sci. U.S.A.* 1991 Oct 1; **88** (19): 8691-8695.

Jiang et al. A novel peptide isolated from a phage display peptide library with trastuzumab can mimic antigen epitope of HER-2. *Journal of Biological Chemistry.* 2005 Feb 11; **280** (6): 4656-4662.

Skolnick et al. From genes to protein structure and function: novel applications of computational approaches in the genomic era. *Trends in Biotechnology* 2000 Jan; **18** (1): 34-39.

Gura T. Systems for identifying new drugs are often faulty. *Science.* 1997 Nov 7; **278** (5340): 1041-1042.

Jain RK. Barriers to drug delivery in solid tumors. *Scientific American.* 1994 Jul; **271** (1): 58-65.

Curti BD. Physical barriers to drug delivery in tumors. *Critical Reviews in Oncology/Hematology.* 1993 Feb; **14** (1): 29-39.

An et al. Cloning and characterization of UROC28, a novel gene overexpressed in prostate, breast, and bladder cancers. *Cancer Research* 2000 Dec 15; **60** (24): 7014-7020.

Vitetta et al. Monoclonal antibodies as agonists: an expanded role for their use in cancer therapy. *Cancer Research.* 1994 Oct 15; **54** (20): 5301-5309.

Bodey et al. Failure of cancer vaccines: the significant limitations of this approach to immunotherapy. *Anticancer Research* 2000 Jul-Aug; **20** (4): 2665-2676.

Chen et al. Discordant protein and mRNA expression in lung adenocarcinomas. *Molecular & Cellular Proteomics* 2002 Apr; **1** (4): 304-313.

Maddala et al. alpha-Crystallin localizes to the leading edges of migrating lens epithelial cells. *Exp. Cell Res.* 2005 May 15; **306** (1): 203-215.

Takizawa et al. Thin is better!: ultrathin cryosection immunocytochemistry. *J. Nippon Med. Sch.* 2004 Oct; **71** (5): 306-307.

(9) Grounds of Rejection

The following ground(s) of rejection are applicable to the appealed claims:

Claim Rejections - 35 USC § 112

The following is a quotation of the first paragraph of 35 U.S.C. 112:

> The specification shall contain a written description of the invention, and of the manner and process of making and using it, in such full, clear, concise, and exact terms as to enable any person skilled in the art to which it pertains, or with which it is most nearly connected, to make and use the same and shall set forth the best mode contemplated by the inventor of carrying out his invention.

A. Claims 78-82, 86-91, and 94 are rejected under 35 U.S.C. 112, first paragraph, as failing to comply with the written description requirement. The claim(s) contains subject matter which was not described in the specification in such a way as to reasonably convey to one skilled in the relevant art that the inventor(s), at the time the application was filed, had possession of the claimed invention.

This is a "written description" rejection.

As first considered, claims 78-82, 86-91, and 94 were drawn to a method for treating cancer comprising administering a member of a genus of "inhibitors" of a peptide or polypeptide.

As explained in the first Office action on the merits of the application, which was mailed November 22, 2004, the subject matter of the claims is not described in a manner that would reasonably convey to the skilled artisan that Appellant had possession of the claimed invention at the time the application was filed, since, in particular, <u>the specification does not include a description of an activity or function of the polypeptide encoded by the nucleic acid molecules of SEQ ID NO: 83 and SEQ ID NO: 85</u>, which can be inhibited, and accordingly, it also fails to describe an inhibitor of such a peptide or polypeptide, which can be administered to a patient to treat cancer in the patient.

The specification describes the polynucleotides of SEQ ID NO: 83 and SEQ ID NO: 85 as novel (page 111, Table 3). While disclosing that both polynucleotides encode the same protein (page 115, lines 11-15), <u>the specification does not describe a function or activity of this protein</u>, and apart from disclosing some results of a preliminary analysis of the amino acid sequence (page 117, lines 19-27), merely describes the protein as localized on the cell membrane of epithelial cells (page 117, lines 10-14).

The description of the claimed invention would therefore not be sufficiently detailed to reasonably convey to the skilled artisan that Appellant had possession of the claimed invention at the time the application was filed, because the skilled artisan could

not immediately envision, recognize, or distinguish an inhibitor suitable for use in practicing the claimed invention.

For example, while the inhibitor could be a monoclonal antibody that binds the protein, because the activity or function of the protein has not been described, an antibody that binds to the protein and thereby inhibits its activity or function cannot be immediately envisioned, recognized, or distinguished, given only the description of the claimed invention set forth in the specification. An antibody that binds the polypeptide may not inhibit its function, as the antibody may be agonist or otherwise, the antibody may not affect the activity of the polypeptide. Consequently, the skilled artisan cannot envision an inhibitor of an activity or function that has not been described, nor could the skilled artisan distinguish a compound capable of inhibiting the activity or function in the absence of such a description. The specification does not reasonably convey Appellant's possession of the claimed invention, due to the lack of an adequate written description of the function that is inhibited by the members of the genus of "inhibitors" that are administered to the patient.

In point of fact, the specification discloses that inhibitors of the protein encoded by the newly identified polynucleotides of SEQ ID NO: 83 and SEQ ID NO: 85 "could [...] potentially be designed" (page 74, lines 3 and 4); however, the specification discloses that doing this would be "complicated by the fact that no specific function has been identified for [...] these gene products, and no data is available on their three-dimensional structures" (page 74, lines 4-6). Because a specific function or activity of the protein encoded by the polynucleotides of SEQ ID NO: 83 and SEQ ID NO: 85 has

not been described, the skilled artisan could not envision, recognize, or distinguish an inhibitor of the protein, and therefore the instant disclosure of the claimed invention cannot be considered sufficient to meet the written description requirement set forth under 35 USC § 112, first paragraph.

The Guidelines for Examination of Patent Applications Under the 35 U.S.C. 112, paragraph 1, ``Written Description" Requirement (66 FR 1099-1111, January 5, 2001) state, "[p]ossession may be shown in a variety of ways including description of an actual reduction to practice, or by showing the invention was 'ready for patenting' such as by disclosure of drawings or structural chemical formulas that show that the invention was complete, or by describing distinguishing identifying characteristics sufficient to show that the applicant was in possession of the claimed invention" (*Id.* at 1104).

The members of the genus of inhibitors of the polypeptide encoded by SEQ ID NO: 83 and SEQ ID NO: 85 are expected to vary markedly in both structure and function (i.e., mode of action), depending upon the function of the polypeptide that is inhibited. The *Guidelines* further state, "[f]or inventions in an unpredictable art, adequate written description of a genus which embraces widely variant species *cannot* be achieved by disclosing only one species within the genus" (Id. at 1106). It follows that an adequate written description of a genus cannot be achieved *in the absence of a disclosure of at least one* species within the genus.

Because the claims are directed to the use of a genus of variant species of inhibitor, an adequate written description of the claimed invention must include sufficient description of at least a representative number of different species of inhibitor by actual

reduction to practice, reduction to drawings, or by disclosure of relevant, identifying characteristics sufficient to show that Appellant was in possession of the claimed genus. Factual evidence of an actual reduction to practice has not been disclosed by Appellant in the specification; nor has Appellant shown the invention was "ready for patenting" by disclosure of drawings or structural chemical formulas that show that the invention was complete; nor has Appellant described distinguishing identifying characteristics sufficient to show that Appellant had possession of the claimed invention at the time the application was filed. Accordingly, the disclosure of the claimed invention fails to meet the written description requirement set forth under 35 USC § 112, first paragraph.

Following receipt of the first Office action on the merits mailed November 24, 2004, Appellant filed the response of April 21, 2005, in which this ground of rejection was traversed in view of the accompanying amendment to the claims.

Appellant's arguments at pages 8-10 of the amendment filed April 21, 2005, were carefully considered but not found persuasive for the following reasons:

As explained in the Final Office action mailed November 2, 2005, the considerations that are made in determining whether a claimed invention is supported by an adequate written description are outlined by the published Guidelines for Examination of Patent Applications Under the 35 U.S.C. 112, para. 1, ``Written Description" Requirement (Federal Register; Vol. 66, No. 4, January 5, 2001). A copy of this publication can be viewed or acquired on the Internet at the following address: <http://www.gpoaccess.gov/>.

These guidelines state that rejection of a claim for lack of written description, where the claim recites the language of an original claim should be rare. Nevertheless, these guidelines further state, "the issue of a lack of written description may arise even for an original claim when an aspect of the claimed invention has not been described with sufficient particularity such that one skilled in the art would recognize that the applicant has possession of the claimed invention" (*Id.* at 1105).

In this instance, claims 78, 86, 87, and 94 are drawn to a method for treating cancer comprising administering a member of a genus of "agents" that bind to a peptide or polypeptide. Claims 79-82 and 88-91 are drawn to the methods of claims 78 or 87, wherein the "agent" is an antibody, as opposed to claims 83, 84, 92, and 93, which are drawn to the methods of claims 78 or 87, wherein the agent is an antibody that is conjugated or linked to a radionuclide or chemotherapeutic agent.

Ipsis verbis support for the term "agent" is found throughout the specification, including the claims, as originally filed; however, the Federal Circuit has explained that *in ipsis verbis* support for the claims in the specification does not *per se* establish compliance with the written description requirement:

> Even if a claim is supported by the specification, the language of the specification, to the extent possible, must describe the claimed invention so that one skilled in the art can recognize what is claimed. The appearance of mere indistinct words in a specification or a claim, even an original claim, does not necessarily satisfy that requirement. The disclosure must allow one skilled in the art to visualize or recognize the identity of the subject matter purportedly described. *Eli Lilly*, 119 F.3d at 1568, 43 USPQ2d at 1406.

Regents of the University of California v. Eli Lilly & Co., 119 F.3d 1559, 43 USPQ2d 1398 (Fed. Cir. 1997). *See also:* University of Rochester v. G.D. Searle & Co., 69 USPQ2d 1886 1892 (CA FC 2004).

While the "agent" to which the claims are directed could be an immunoconjugate comprising an antibody that binds the protein and either a radionuclide or chemotherapeutic agent, the specification discloses that this genus of "agents" includes, for example, specific "inhibitors" of the polypeptide encoded by the nucleic acid sequences of SEQ ID NO: 83 or SEQ ID NO: 85. Thus, the genus of "agents" includes, for example, naked antibodies that bind the polypeptide and inhibits its specific activity or function. Moreover, while the genus of "agents" that inhibit the activity or function of the polypeptide includes such antibodies, the specification discloses the genus includes "other inhibitors" (page 18, lines 18-20), which are thereafter not described in any additional detail. So, besides antibodies, this genus of "agents" that specifically bind to the polypeptide and inhibit its activity or function includes, for example, peptides and small molecules. Notably, such peptides and small molecules bare no apparent structural or functional relationship to antibodies that bind the polypeptide.

Thus, giving the broadest, reasonable interpretation, the claims are directed to a genus of "agents", which include naked antibodies, antibodies conjugated to radionuclides or chemotherapeutic agents, and other substances, which vary both structurally and functionally, despite their common ability to bind to the polypeptide encoded by the nucleotide sequences set forth as SEQ ID NO: 83 or SEQ ID NO: 85 and/or inhibit its function.

As explained in the preceding Office action, although the specification describes the polynucleotides of SEQ ID NO: 83 and SEQ ID NO: 85 as novel (page 111, Table 3), it does not disclose the specific activities or functions of the polypeptides encoded by these nucleotide sequences, which are inhibited by members of the genus of "agents" to which the claims are directed. Consequently, as further explained in the preceding Office action, the skilled artisan cannot envision such agents, which are inhibitors of an activity or function that has not been described, nor could the skilled artisan distinguish a compound capable of inhibiting the activity or function in the absence of such a description. Therefore, the specification would not reasonably convey to the skilled artisan that Appellant had possession of the claimed invention at the time the application was filed.

Although Appellant argued that the presently claimed invention is adequately described, the genus of "agents", which includes antibodies, is not limited to antibodies that are conjugated to radionuclides or chemotherapeutic agents, which bind the polypeptide encoded by SEQ ID NO: 83 or SEQ ID NO: 85. Instead, the genus includes structurally and functionally disparate molecules, including, for example, naked antibodies, that specifically bind to the polypeptide and inhibit its activity or function, such that treatment of cancer cells with the antibody provides therapeutic benefit.

Again, as explained previously, an antibody that binds the polypeptide <u>may not inhibit its function</u>, as the antibody may be agonist or otherwise, the antibody may not affect the activity of the polypeptide. Only an antibody that binds the polypeptide, *which*

is conjugated to a radionuclide or chemotherapeutic agent, could be immediately envisioned, given the otherwise inadequate disclosure of the claimed invention.

However, because a specific function or activity of the protein encoded by the polynucleotides of SEQ ID NO: 83 and SEQ ID NO: 85 has not been described, the skilled artisan could not envision, recognize, or distinguish an inhibitor, including an antibody, of the protein.

"[G]eneralized language may not suffice if it does not convey the detailed identity of an invention." *University of Rochester v. G.D. Searle Co.*, 69 USPQ2d 1886 1892 (CAFC 2004). In this instance, there is no language that adequately describes the genus of antibodies that bind the polypeptide and inhibit its activity or function, so as to provide therapeutic benefit. A description of what a material does, rather than of what it is, does not suffice to describe the claimed invention.

Notably the Federal Circuit has recently decided that the description of a fully characterized molecular target of an antibody is sufficient to adequately describe an antibody that binds that target. See *Noelle v. Lederman*, 69 USPQ2d 1508 (CA FC 2004). However, the same court decided that each case involving the issue of written description, "must be decided on its own facts. Thus, the precedential value of cases in this area is extremely limited." *Vas-Cath*, 935 F.2d at 1562 (quoting *In re Driscoll*, 562 F.2d 1245, 1250 (C.C.P.A. 1977)).

Following the example set by the Federal Circuit in deciding *Noelle v. Lederman*, then, were the claims directed to an antibody that binds a well-characterized antigen, the written description would be met. However, the claims are not solely directed to an

antibody that binds a well-characterized molecular target, but rather to a naked antibody that binds a polypeptide and inhibits its activity or function, so as to be therapeutically effective; and yet, the specification fails to describe the activity or function of the polypeptide. The specification fails to describe an antibody that binds the polypeptide to specifically inhibit its activity or function; and it fails to describe an antibody not conjugated to a radionuclide or chemotherapeutic agent, which inhibits the progression of breast, bladder or prostate cancer. Moreover, it fails to describe the "epitope" of the polypeptide to which such an inhibitory antibody must bind.

There is factual evidence that the detailed description of an antigen, as opposed to the detailed description of an epitope of an antigen, should not always be regarded as sufficient to describe the antibody that binds that antigen, particularly in instances where binding of the antibody modulates the activity of the antigen. For example, Stancoviski et al. *(Proceedings of the National Academy of Science USA.* 1991; **88**: 8691-8695) characterized the binding effects upon the growth of tumor cells of different antibodies, each of which bind different epitopes of the extracellular domain of a tumor-associated antigen related to EGFR, namely ErbB2; see entire document (e.g., the abstract). Stancovski et al. teaches some anti-ErbB2 antibodies inhibited tumor cell growth, but others actually accelerated their growth (page 8693, column 1). By way of explanation, Jiang et al. (*J. Biol. Chem.* 2005 Feb 11; **280** (6): 4656-4662) teaches that it is well known that different biological effects are associated with epitope specificity of the antibodies; see entire document, particularly page 4656, column 2.

Accordingly, the mere generalized description of antibodies that bind a well-characterized antigen, as opposed to a well-characterized epitope of an antigen, cannot always suffice to describe adequately antibodies that have, for example, an inhibitory or therapeutic effect, because the skilled artisan could not immediately envision, recognize, or distinguish those antibodies that bind an antigen on tumor cells and inhibit the growth of those tumor cells from antibodies that bind the antigen but lack therapeutic effect (e.g., promote the growth of tumor cells).

Furthermore, it is aptly noted that the Federal Circuit has decided that a generic statement that defines a genus of substances by *only* their functional activity, i.e., the ability to specifically bind a polypeptide and inhibit its activity, or the ability to bind a cancer cell and inhibit its growth or metastatic progression, <u>does not provide an adequate written description of the genus</u>. <u>See</u> *The Reagents of the University of California v. Eli Lilly*, 43 USPQ2d 1398 (CAFC 1997). The Court indicated that while not required to disclose every species encompassed by a genus, the description of a genus is achieved by the recitation of a precise definition of a representative number of members of the genus, such as by reciting the structure, formula, chemical name, or physical properties of those members, rather than by merely reciting a wish for, or even a plan for obtaining a genus of molecules having a particular functional property. The recitation of a functional property alone, which must be shared by the members of the genus, is merely descriptive of what the members of genus must be capable of doing, not of the substance and structure of the members.

Although *Lilly* related to claims drawn to genetic material, the statute applies to all types of inventions. "Regardless whether a compound is claimed *per se* or a method is claimed that entails the use of the compound, the inventor cannot lay claim to the subject matter unless he can provide a description of the compound sufficient to distinguish infringing compounds from non-infringing compounds, or infringing methods from non-infringing methods". *University of Rochester v. G.D. Searle Co.*, 69 USPQ2d 1886 1984 (CAFC 2004). Without the "agents" (e.g., naked antibodies that bind the polypeptide and inhibit its activity, so as to be therapeutically effective) to which the claims are directed, it is impossible to use the claimed invention.

In addition, although the skilled artisan could potentially screen candidate agents (e.g., antibodies) to identify those that bind the polypeptide and inhibit its function, so as to be therapeutically effective in treating breast, bladder or prostate cancer, it is duly noted that the written description provision of 35 U.S.C § 112 is severable from its enablement provision; and adequate written description requires more than a mere statement that it is part of the invention and reference to a potential method for isolating it.

> The purpose of the "written description" requirement is broader than to merely explain how to "make and use"; the applicant must convey with reasonable clarity to those skilled in the art that, as of the filing date sought, he or she was in possession *of the invention*. The invention is, for purposes of the "written description" inquiry, *whatever is now claimed*.

Vas-Cath, Inc. v. Mahurkar, 935 F.2d 1555, 1563-64, 19 USPQ2d 1111, 1117 (CAFC 1991). <u>See</u> *Fiers v. Revel*, 25 USPQ2d 1601, 1606 (CAFC 1993); *Amgen Inc. v.*

Chugai Pharmaceutical Co. Ltd., 18 USPQ2d 1016 (CAFC 1991); *University of Rochester v. G.D. Searle Co.*, 69 USPQ2d 1886 1892 (CAFC 2004).

Thus, contrary to Appellant's arguments, the disclosure fails to satisfy the "written description" requirement set forth under 35 U.S.C. § 112, first paragraph.

B. Claims 78-84 and 86-94 are rejected under 35 U.S.C. 112, first paragraph, as failing to comply with the enablement requirement. The claim(s) contains subject matter which was not described in the specification in such a way as to enable one skilled in the art to which it pertains, or with which it is most nearly connected, to make and/or use the invention.

As first considered, claims 78-84 and 86-94 were drawn to a method for treating cancer cells in a patient comprising administering to the patient an inhibitor of the polypeptide encoded by SEQ ID NO: 83 or SEQ ID NO: 85, including an antibody that binds the polypeptide.

As explained in the first Office action on the merits of the application, which was mailed November 22, 2004, the amount of guidance, direction, and exemplification set forth in the specification would not be sufficient to enable the skilled artisan to use the claimed invention without undue experimentation.

The specification teaches SEQ ID NO: 3 is the polynucleotide sequence of an expressed sequence tag (EST), which is abundantly expressed, compared to normal, in prostate, breast, and bladder cancer; see, e.g., Figures 3, 15, and 16. The specification teaches that the polynucleotide of SEQ ID NO: 3 was used as a probe to select the full-

length complementary DNA (cDNA) molecules of SEQ ID NO: 83 and SEQ ID NO: 85, which were derived from two alternatively spliced messenger RNA (mRNA) variants encoding the same protein having the amino acid sequence set forth as SEQ ID NO: 84 and SEQ ID NO: 86 (page 115, lines 6-15).

The specification teaches these isolated polynucleotides and the polypeptide encoded by the polynucleotides were not previously known or described (page 111, Table 3). The specification does not teach what function or biologic activity the polypeptide has; yet, the claimed invention is a method for treating cancer, which comprises administering to a patient an inhibitor of the polypeptide. Because the function or activity of the polypeptide is not disclosed, the skilled artisan could not use the claimed invention without first having to perform an undue amount of additional experimentation to first determine the function or activity of the protein, secondly to determine whether the function or activity of the protein correlates with the onset or progression of cancer, and if so, then to design or discover a compound that inhibits that function or activity, which can be used in practicing the claimed invention to treat cancer.

The specification discloses, "identification of protein function can be extrapolated, in some cases, from primary sequence data, provided that sequence homology exists between the unknown protein and a protein of similar sequence and known function" (page 71, lines 7-9). However, the specification does not teach whether the polypeptide encoded by the polynucleotide sequences of SEQ ID NO: 83 and SEQ ID NO: 85 is homologous to proteins having known functions; and nevertheless, Skolnick et al.

(*Trends in Biotechnology* 2000; **18**: 34-39), for example, discloses that the skilled artisan is well aware that assigning functional activities for any particular protein or protein family based upon sequence homology is inaccurate, in part, because of the multifunctional nature of proteins (see, e.g., the abstract; and page 34, *Sequence-based approaches to function prediction*). Skolnick et al. teaches that even in situations where there is some confidence of a similar overall structure between two proteins, only experimental research can confirm the artisan's best guess as to the function of the structurally related protein (see, in particular, the abstract and Box 2). Thus, contrary to the assertions set forth in the instant disclosure, the skilled artisan cannot reliably and accurately predict the function of a novel protein upon the basis of only an observed similarity in its amino acid sequence and those of other proteins having known functions.

Even if the function or activity of the polypeptide were known, the skilled artisan could still not use the claimed invention without first having to perform an undue amount of additional experimentation, because <u>the specification does not teach the skilled artisan to make an inhibitor of the polypeptide, which, in particular, can be used in practicing the claimed invention to treat cancer</u>. Additionally, even though cancer cells may overexpress the protein, its function or activity may not be associated with the onset or progression of cancer; therefore, an inhibitor of the polypeptide may not inhibit the onset or progression of cancer in the patient and would therefore not provide an effective treatment of cancer. Consequently, before designing or striving to discover an

inhibitor of the protein, the skilled artisan would have to determine if such an inhibitor might be therapeutically valuable.

Even if the activity of the protein were known to be associated with the onset or progression of cancer, the art of anticancer drug discovery is unfortunately hindered by the extreme complexity of the biological system and its inherently unpredictable nature and consequently an inhibitor of the polypeptide could not be made by routine experimentation alone. For example, Gura (*Science* 1997; **278**: 1041-1042) teaches that researchers face the problem of sifting through potential anticancer agents to find ones promising enough to make human clinical trials worthwhile (abstract). Gura teaches that since formal screening began in 1955, many thousands of drugs have shown activity in either cell or animal models, but that only 39 have actually been shown to be useful for chemotherapy (page 1041, first and second paragraphs). Because of the lack of predictability in the art, Gura discloses that often researchers merely succeed in developing a therapeutic agent that is useful for treating the animal or cell that has been used as a model, but which is ineffective in humans, indicating that the results acquired during pre-clinical studies are often non-correlative with the results acquired during clinical trials (page 1041, column 2).

Furthermore, because of the refractory nature of cancer to drugs, the design and discovery of effective drugs can be, and usually is, daunting. Jain (*Scientific American* 1994; **271**: 58-65), for example, teaches that most tumors resist full penetration by anticancer agents (page 58, column 1) and that scientists need to put expanded effort into uncovering the reasons why therapeutic agents that show encouraging promise in

the laboratory often turn out to be ineffective in the treatment of common solid tumors (page 65, column 3). Curti (*Critical Reviews in Oncology/Hematology* 1993; **14**: 29-39) teaches that solid tumors resist destruction by chemotherapy agents and that although strategies to overcome defense mechanisms of neoplastic cells have been developed and tested in a number of patients, success has been limited. Curti discloses that our knowledge about the physical barriers to drug delivery in tumors is a work in progress (page 36, column 2). Curti teaches that it is certainly possible that cancer cells possess many as yet undefined additional molecular mechanisms to defeat chemotherapy treatment strategies and, if this is true, designing effective chemotherapeutic regimens for solid tumors may prove a burdensome task (paragraph bridging pages 29-30).

Anti-tumor agents must accomplish several tasks to be effective. The agents must be delivered into the circulation that supplies the tumor and interact at the proper site, and they must do so at a sufficient concentration and for a sufficient period of time so as to be effective. Also, the targeted cells must not have an alternate means of survival despite action at the proper site for the drug. In addition, variables such as biological stability, half-life, and clearance from the blood are important parameters in achieving successful therapy. The composition may be inactivated *in vivo* before producing a sufficient effect, for example, by degradation, immunological activation, or due to an inherently short half-life. The composition may not otherwise reach the target because of its inability to penetrate tissues or cells where its activity is to be exerted. Alternatively, the composition may be absorbed by fluids, cells and tissues where the

formulation has no effect and circulation into the target area may be insufficient to carry the composition and to permit a large enough local concentration to be established.

It is noted that the claims specifically encompass a method for treating cancer, which comprises administering to a patient an antibody that binds the polypeptide; however, while the specification teaches the protein has been localized to epithelial cells, "mainly on the cell membrane" (page 117, lines 10-14), the specification does not actually teach whether the protein is expressed at the surface of the cells. If the inhibitor is an antibody or another type of inhibitor that binds directly to the polypeptide, and the polypeptide is not expressed at the surface of the targeted cancer cells, the antibody or other inhibitor cannot specifically bind those cells and therefore will have no specific inhibitory effect upon those cells. Accordingly, an undue amount of additional experimentation would be necessary to determine if the protein is expressed at the surface of cancer cells before the skilled artisan could use the claimed invention to treat cancer in a patient.

In this regard, it appears that An et al. (*Cancer Research* 2000; **60**: 7014-7020) teaches the gene encoding the protein, which is designated therein as UROC28, is not expressed at the surface of cells, since immunohistochemical analyses of glandular epithelial cells of prostate and breast cancers revealed the protein localizes in the nucleus and cytoplasm; see entire document, particularly page 7018, Figure 5. Furthermore, An et al. discloses the presence of the protein in human serum specimens acquired from patients diagnosed with prostate cancer (see, e.g., page 7018, figure 6). All together, An et al. suggests that, rather than expressed at the surface of cells of

epithelial origin, the protein is either nuclear or cytoplasmic, or both, and can be secreted. Again, if the protein is not expressed at the surface of targeted cancer cells, however, an antibody or other inhibitor that binds directly to the protein cannot be used, because the antibody or other inhibitor cannot bind a protein that is expressed within the cell, and if the protein is secreted, while the antibody or inhibitor could bind the protein, its binding to the protein will not affect the cancer cells that secreted the protein.

Even if the protein were expressed at the surface of the targeted cancer cells, and the inhibitor is an antibody that binds the protein, it is aptly noted that, while antibody-targeted therapy can overcome some of the intrinsic shortcomings that reduce the efficacy of agents that are non-selective or non-tumor-specific, there are well known limitations in the art of antibody-targeted therapeutic regimens. Vitetta et al. (*Cancer Research* 1994; **54**: 5301-5309) teaches: "[D]espite [...] intellectual appeal, the general therapeutic efficacy of tumor-reactive MAbs [monoclonal antibodies] has been disappointing. In particular the results of clinical studies in patients with solid tumors showed little efficacy, except in the setting of minimal disease" (citations omitted) (page 5301, column 1). Vitetta et al. teaches that there are a number of significant limitations in their use as first-line therapy for solid tumors page 5305, (columns 1-2):

> Only 0.001 to 0.1% of injected MAb [monoclonal antibody] will localize to each [gram] of tumor mass. Moreover, MAbs, even at high serum concentrations, cannot gain access to all the cells in solid epithelial tumor. The reasons for this are poor and heterogeneous blood supply, the blood-tumor barrier, and the selective binding of the MAb by the tumor cells closest to the blood supply. In addition, MAbs by themselves probably cannot kill the 10^{10}-10^{12} malignant cells that may be necessary to cure a patient with a disseminated tumor (citations omitted) (page 5305, columns 1-2).

The strategic approach to treating cancer using antibody therapy is analogous to active specific immunotherapy (e.g., vaccination against tumor-associated antigens), at least to the extent that the latter theoretically induces a humoral immune response (i.e., the production of tumor-specific antibody). Antibody therapy can be defined as passive immunization, cancer vaccine therapy as active immunization. Because the efficacy of both approaches depends upon the effectiveness of tumor antigen-specific antibodies to ameliorate or inhibit tumors, both also share the same or corresponding limitations. Bodey et al. (*Anticancer Research* 2000; **20**: 2665-2676) teaches:

> Animal models, albeit highly artificial, have yielded promising results. Clinical trials in humans, however, have been somewhat disappointing. Although general immune activation directed against the target antigens contained with a cancer vaccine has been documented in most cases, reduction in tumor load has not been frequently observed, and tumor progression and metastasis usually ensue, possibly following a slightly extended period of remission. The failure of cancer vaccines to fulfill their promise is due to the very relationship between host and tumor: through a natural selection process the host leads to the selective enrichment of clones of highly aggressive neoplastically transformed cells, which apparently are so dedifferentiated that they no longer express cancer cell specific molecules. Specific activation of the immune system in such cases only leads to lysis of the remaining cells expressing the particular TAAs [tumor associated antigens] in the context of the particular human leukocyte antigen (HLA) subclass and the necessary costimulatory molecules. The most dangerous clones of tumor cells however lack these features and thus the cancer vaccine is of little use.

Accordingly, as noted above, if a cancer cell does not express the protein that is specifically bound by the antibody at its surface, the use of a pharmaceutical composition comprising such an antibody will not be effective; but, in addition, as Bodey et al. teaches, the use of such a pharmaceutical composition may paradoxically serve to

select against tumor cells that express the protein, while promoting the growth of tumor cells that do not express the protein.

It is further noted that the specification does not actually teach that the polypeptide of SEQ ID NO: 84 and SEQ ID NO: 86, which is expressed by the polynucleotides of SEQ ID NO: 83 or SEQ ID NO: 85, is over-expressed in cancer cells, compared to normal cells of the same tissue type. Moreover, <u>the specification fails to demonstrate a correlation between the level of mRNA expression and the level of protein expression in cancer cells</u>. One cannot presume that the amount of protein produced in a cell will mirror the amount of mRNA produced, since Chen et al. (*Molecular & Cellular Proteomics* 2002; **1**: 304-313), for example, teaches that the expression levels protein and mRNA in cancer cells are discordant; see entire document (e.g., the abstract). Moreover, Lewin has written: "But having acknowledged that control of gene expression can occur at multiple stages, *and that production of RNA cannot inevitably be equated with production of protein*, it is clear that the overwhelming majority of regulatory events occur at the initiation of transcription" (italicized for emphasis) (<u>Genes VI</u>, 1997; Ed. Benjamin Lewin; Chapter 29, first page). If the protein is expressed at the surface of cells, and the inhibitor is an antibody, unless the cancer cells, relative to normal cells of the same tissue type, more abundantly express the protein, the antibody will not selectively target cancer cells, but will also undesirably target normal cells. One skilled in the art could therefore not use the claimed invention without first performing an undue amount of additional experimentation to determine if the protein encoded by the polynucleotides of SEQ ID NO: 83 and SEQ ID NO: 85 is

over-expressed in prostate, breast, and bladder cancer cells, compared to normal cells of the same tissue type.

Finally, while the claims are drawn to a method for treating any type of cancer, the specification teaches that the polynucleotides of SEQ ID NO: 83 and SEQ ID NO: 85 are <u>not abnormally expressed in colon or lung cancer</u>; see, e.g., Figure 15. Thus, the specification shows that the skilled artisan cannot predict which types of cancer will express the protein, or to what extent; so an undue amount of additional experimentation would have to be performed to characterize the expression of the polypeptide by other types of cancer before the claimed invention could be used to treat cancer in a patient.

In conclusion, upon careful consideration of the factors used to determine whether undue experimentation is required, in accordance with *Ex parte Forman*, 230 USPQ 546 (BPAI 1986), the amount of guidance, direction, and exemplification disclosed by Appellant is not deemed sufficient to enable the skilled artisan to use the claimed invention without a need to perform an undue amount of additional experimentation.

Following receipt of the first Office action on the merits mailed November 24, 2004, Appellant filed the response of April 21, 2005, in which this ground of rejection was traversed in view of the accompanying amendment to the claims.

Appellant's arguments at pages 10-13 of the amendment filed April 21, 2005, were carefully considered but not found persuasive for the following reasons:

As explained in the Final Office action mailed November 2, 2005,

M.P.E.P. § 2164.01 states:

> The standard for determining whether the specification meets the enablement requirement was cast in the Supreme Court decision of *Mineral Separation v. Hyde*, 242 U.S. 261, 270 (1916) which postured the question: is the experimentation needed to practice the invention undue or unreasonable? That standard is still the one to be applied. *In re Wands*, 858 F.2d 731, 737, 8 USPQ2d 1400, 1404 (Fed. Cir. 1988). Accordingly, even though the statute does not use the term "undue experimentation," it has been interpreted to require that the claimed invention be enabled so that any person skilled in the art can make and use the invention without undue experimentation. *In re Wands*, 858 F.2d at 737, 8 USPQ2d at 1404 (Fed. Cir. 1988).

There are many factors to be considered when determining whether there is sufficient evidence to support a determination that a disclosure does not satisfy the enablement requirement and whether any necessary experimentation is "undue". These factors, which have been outlined in the Federal Circuit decision of *In re Wands*, 858 F.2d 731, 737, 8 USPQ2d 1400, 1404 (Fed. Cir. 1988), include, but are not limited to, the nature of the invention, the state of the prior art, the relative skill of those in the art, the amount of direction or guidance disclosed in the specification, the presence or absence of working examples, the predictability or unpredictability of the art, the breadth of the claims, and the quantity of experimentation which would be required in order to practice the invention as claimed. See also *Ex parte Forman*, 230 USPQ 546 (BPAI 1986).

Careful consideration of these factors indicates that the amount of guidance, direction, and exemplification disclosed in the specification, as filed, would not be

sufficient to have enabled the skilled artisan to use the claimed invention at the time the application was filed without undue and/or unreasonable experimentation.

As explained in the "written description" rejection above, the claims are drawn to a method for treating cancer comprising administering a member of a genus of "agents" that bind to a peptide or polypeptide, so as to be therapeutically effective. While the "agent" could be an antibody that binds the protein and which is conjugated to a radionuclide or chemotherapeutic agent, the specification discloses that this genus of "agents" includes "inhibitors" of the polypeptide encoded by the nucleotide sequence of SEQ ID NO: 83 or SEQ ID NO: 85. As explained in the preceding Office action, although the specification describes SEQ ID NO: 83 and SEQ ID NO: 85 as novel (page 111, Table 3), it does not teach the specific activity or function of the polypeptide encoded by these nucleic acid sequences. Because the function or activity of the polypeptide is not disclosed, the skilled artisan could not use the claimed invention without first having to perform undue and unreasonable additional experimentation to first determine the function or activity of the protein, secondly to determine whether the function or activity of the protein correlates with the onset or progression of cancer, and if so, then to design or discover a compound that inhibits that function or activity, which can be used in practicing the claimed invention to treat breast, bladder or prostate cancer.

Although the specification asserts it is possible to predict protein function, in some cases, from primary sequence data, provided that sequence homology exists between the unknown protein and a protein of similar sequence and known function, in

this instance, the specification does not disclose whether the polypeptide encoded by SEQ ID NO: 83 or SEQ ID NO: 85 bear any significant and substantial homology to other proteins of known functions and activities. Moreover, as evidenced by Skolnick et al. (of record), for example, the skilled artisan is well aware that assigning functional activities for any particular protein or protein family based upon sequence homology is inaccurate. Thus, contrary to the assertions set forth in the instant disclosure, the skilled artisan cannot reliably and accurately predict the function of a novel protein upon the basis of only an observed similarity in its amino acid sequence and those of other proteins having known functions.

In fact, the specification, itself, supports this very position, since it discloses, "Inhibitors could also potentially be designed for the previously unreported prostate, bladder or breast cancer-markers identified in the present invention [but this] is complicated by the fact that no specific function has been identified for most of these gene products, and no data is available on their three-dimensional structures" (page 71, lines 1-6).

It was further noted in the preceding Office action that even if the function or activity of the polypeptide were known, the skilled artisan could still not use the claimed invention without first having to perform undue and unreasonable experimentation, because <u>the specification does not teach the skilled artisan to make an inhibitor of the polypeptide, which, in particular, can be used in practicing the claimed invention to treat cancer</u>. Additionally, even though cancer cells may overexpress the protein, its function or activity may not be associated with the onset or progression of cancer; therefore, an

inhibitor of the polypeptide may not inhibit the onset or progression of cancer in the patient and would therefore not provide an effective treatment of cancer. Consequently, before designing or striving to discover an inhibitor of the protein, the skilled artisan would have to determine if such an inhibitor might be therapeutically valuable.

Inasmuch as the claims are directed to antibodies, as explained in "written description" rejection above, not all antibodies are reasonably expected to be capable of inhibiting the activity and function of the polypeptide. Other antibodies may not have any effect upon its activity or function, whereas some antibodies may actually act as agonists, promoting or enhancing its activity or function, rather than inhibiting the polypeptide.

Claims 83 and 92 are directed to antibodies that bind the polypeptide, which are conjugated to a radionuclide. In general, an antibody that binds selectively to a cancer cell, which is conjugated to a radionuclide, may be used to treat the disease, since it provides exposure to the radioactivity, which is toxic to the cells. However, as explained in the preceding Office action, while the specification teaches the protein has been localized to epithelial cells, "mainly on the cell membrane" (page 117, lines 10-14), the specification <u>does not actually teach whether the protein is expressed at the surface of the cells</u>. If the inhibitor is an antibody or another type of inhibitor that binds directly to the polypeptide, and the polypeptide is not expressed at the surface of the targeted cancer cells, the antibody or other inhibitor cannot specifically bind those cells and therefore will have no specific inhibitory effect upon those cells. As further noted in the preceding Office action, it is believed that An et al. (of record) provides factual evidence

that the protein, which is designated therein as UROC28, is <u>not expressed at the surface of cells</u>. An et al. teaches immunohistochemical analyses of glandular epithelial cells of prostate and breast cancers revealed <u>the protein localizes in the nucleus and cytoplasm</u>; see entire document, particularly page 7018, Figure 5.

Appellant provided a copy of a declaration under 37 C.F.R. § 1.132 by Dr. Veltri, which was filed during prosecution of a copending, related application (i.e., U.S. Patent Application No. 09/966,762). Appellant argued that this declaration provides factual evidence that the polypeptide (i.e., UROC28), or at least a portion thereof, is present at the extracellular surface of prostate cancer cells.

The merit of the declaration filed in the copending application was carefully considered to the extent that it is believed to be applicable to the issues raised herein.

Dr. Veltri suggested that An et al. does not teach the protein was *not* expressed as an extracellular, plasma membrane-associated, or trans-membrane protein, only that it was *primarily* localized in the cytoplasm (i.e., the inside of the cell) and more particularly, to at least some extent, in the nucleus; see item #7 at page 3. Agreeably, An et al. does not teach that the protein, or a portion thereof, was *not* exposed at the cell's extracellular surface; but, if it was, its presence or level of expression at the extracellular surface must not have been remarkable.

The declaration states, "the present specification discloses [...] a significant of UC28 localizes to the cell membrane" (item #9 at page 3). There is, however, no factual evidence attached to the declaration to support this assertion. As explained in the preceding Office action, while the instant specification teaches the protein has been

localized to epithelial cells, "mainly on the cell membrane" (page 117, lines 10-14), the specification <u>does not actually teach whether the protein is expressed at the surface of the cells</u>. Given the methodology and resolution of the microscopy used by An et al. it is submitted that it would not be possible to reasonably conclude that the protein is exposed at the surface of the cell; if such, methodology was used by Appellant to determine that the protein is "mainly on the cell membrane", it is further submitted that, using such methodology, one could not reliably distinguish a protein that is localized on the inside surface of the plasma membrane from a protein that is localized in the cytoplasm, or a protein that is transmembrane protein. Because the tissue sections used in the process are fixed and permeabilized, the antibody is capable of binding antigens both inside and out. Furthermore, the antibody that was used was a polyclonal antibody, so it recognizes many different antigenic determinants on the protein, not just antigenic determinants present on a putative extracellular domain; therefore, at the resolution used, it would not be possible to determine with any degree of certainty whether the protein is exposed at the surface of living cells.

The specification teaches the protein has been localized to epithelial cells, "mainly on the cell membrane" (page 117, lines 10-14), but as evidenced by Maddala et al. (*Exp. Cell Res.* 2005; **306**: 203-215), for example, not all proteins that appear localized "mainly on the cell membrane" are transmembrane proteins comprising an extracellular domain that is accessible to an antibody at the outside surface of the cell; see entire document (e.g., the abstract). Maddala et al. teaches localization of α-crystallin, an intracellular protein, to the leading edges of the plasma membrane of lens

epithelial cells; see, e.g., the abstract. This protein associates with the plasma membrane, but it is not a transmembrane protein. Using the methodology exemplified by An et al., how might one distinguish a protein, such as α-crystallin from a transmembrane protein, given that both proteins would appear to localize to the plasma membrane? At the resolution used by An et al., it is submitted that such a distinction could not be made with reasonable certainty. The specification provides no factual evidence that suggests that the protein encoded by nucleotide sequences SEQ ID NO: 83 or SEQ ID NO: 85 is a transmembrane protein, as opposed to an intracellular protein that associates with the plasma membrane. The declaration asserts that the protein is accessible to an antibody at the surface of prostate, bladder and breast cancer cells, but provides no factual evidence to support the assertion. An et al., on the other hand, suggests the protein is not a transmembrane protein, but instead a soluble protein localized primarily to the cytoplasm and nucleus.

The declaration further states the use of conventional confocal fluorescence microscopy limited the ability of An et al. to more specifically characterize the localization of the protein and suggests the specification teaches the use of other methodology that remedies the inadequacy of their earlier methodology, so as to have permitted the accurate localization of the protein to outside surface of the plasma membrane. Agreeably, the methodology used by An et al. would not permit one to accurately localize the protein, or a portion thereof, to the outside surface of the cell. As evidenced by, for example, the attached references (i.e., Takizawa et al. (*J. Nippon Med. Sch.* 2004; **71** (5): 306-307) and Maddala et al. (*supra*)), the resolution of the

microscopy used by An et al. would not have permitted such a conclusion. However, contrary to the statement by Dr. Veltri, there does not appear to be any disclosure in the instant specification of the use of high-resolution confocal immunofluorescent microscopy to localize the protein. In fact, the words "confocal" and "microscopy" do not appear in the specification. Thus, if any merit of the declaration is extended to the instant application, it is not known to which disclosures in the instant specification Dr. Veltri would have referred as providing remedy to the inadequacy of the methodology used by An et al.

The declaration notes the presence of a *putative* transmembrane domain in the protein and states, "the presence of this putative transmembrane domain indicates that UC28 localized to the cell membrane" (item #6). If the domain is only a *putative* transmembrane domain, it has not yet been determined to *be* a transmembrane domain. The disclosure by An et al. suggests that the protein is not present at the extracellular surface, as if it were, its presence there was unremarkable. An et al. teaches the protein primarily localized to the inside of the cell (i.e., the cytoplasm and the nucleus); the results disclosed by An et al. do not suggest that the protein comprises an extracellular domain, or that at least part of the protein is exposed at the surface of cancer cells.

Appellant referred to Carrol (Exhibit A); Carrol has written a commentary addressing the importance of findings disclosed by Milowsky et al. that an antibody that specifically binds protate-specific membrane antigen (PMSA), which is radiolabeled, can be used to treat patients with prostate cancer. Notably, Carrol comments that the

antigen to which the antibody binds is an excellent target because it is not secreted like PSA or PAP. As mentioned in the preceding Office action, An et al. suggests that the polypeptide encoded by SEQ ID NO: 83 or SEQ ID NO: 85 (i.e., UROC28) is secreted, as it was detected in serum specimens acquired from patients diagnosed with prostate cancer (see, e.g., page 7018, figure 6). Accordingly, Carrol provides factual evidence that the polypeptide is a less desirable target than PMSA since it is secreted. Furthermore, Carrol emphasizes that the reason that PMSA is an excellent target is that the antibody that recognizes it binds tightly to its extracellular domain, as previous monoclonal antibodies bound to an intracellular domain only accessible in already dead or dying cells. Here, as explained above, it is not known whether the protein comprises an extracellular domain that might serve as the target of an antibody, which is conjugated to a radionuclide. To any extent that Carrol might provide support for Appellant's assertion that the claimed invention can be used without undue and/or unreasonable experimentation, that evidence is merely anecdotal. Again, the antibody or other inhibitor to which the claims are directed cannot effectively bind the protein, if it is present only *within* living cancer cells, and if the protein is secreted, while the antibody or inhibitor could bind the protein, it's binding to the protein will not affect the cancer cells that secreted the protein.

Claims 84 and 93 are directed to antibodies that bind the polypeptide encoded by the nucleotide sequence of SEQ ID NO: 83 or SEQ ID NO: 85, which are linked to chemotherapeutic agents. As evidenced by Vitetta et al. (of record), for example, there are well known limitations in the art of antibody-targeted therapeutic regimens; but, as

explained in the preceding Office action, if a cancer cell does not express the protein that is specifically bound by the antibody at its surface, the use of a pharmaceutical composition comprising such an antibody will not be effective. The specification provides no guidance as to which chemotherapeutic agents are linked to the antibody, so as to provide the claimed therapeutic effect, but it is aptly noted that many chemotherapeutic agents, unlike radionuclides, must gain access to the inside of the cell to cause harm to the cell. Generally, an immunoconjugate comprising such a chemotherapeutic agent binds an antigen at the surface of the cell, which is then "internalized" by the cell; however, not all antigens (e.g., receptors) are "internalized" and thus many antigens to not constitute suitable targets for such therapeutic agents. Not only is it not known that the protein encoded by the nucleotide sequence of SEQ ID NO: 83 or SEQ ID NO: 85 comprises a suitable extracellular domain, but it is also not known whether the protein is "internalized" by the cell following the binding of an antibody.

Furthermore, as Bodey et al. (of record) teaches, the use of such a pharmaceutical composition may paradoxically serve to select against tumor cells that express the protein, while promoting the growth of tumor cells that do not express the protein. It is here again noted that the specification does not teach the activity or function of the polypeptide encoded by the nucleic acid sequence of SEQ ID NO: 83 or SEQ ID NO: 85; and moreover, although the gene encoding the polypeptide is over-expressed, it is not known whether the polypeptide plays an essential role in the life of

the cancer cell, but if it does not, it follows that the use of the claimed invention may lead only to selection against tumor cells that do not express the polypeptide.

Consequently, even if the activity of the protein were known to be essential to the life of the cancer cell, as evidenced by Gura (of record), for example, the art of anticancer drug discovery is unfortunately hindered by the extreme complexity of the biological system and its inherently unpredictable nature. Consequently an inhibitor of the polypeptide (i.e., a naked antibody or "other inhibitor" of the polypeptide) cannot be recognized or made by routine experimentation alone.

It is further noted that the specification does not actually teach that the polypeptide of SEQ ID NO: 84 and SEQ ID NO: 86, which is expressed by the polynucleotides of SEQ ID NO: 83 or SEQ ID NO: 85, is over-expressed in cancer cells, compared to normal cells of the same tissue type. Moreover, <u>the specification fails to demonstrate a correlation between the level of mRNA expression and the level of protein expression in cancer cells</u>.

In response, Appellant argued that An et al. teaches the protein is overexpressed in prostate and breast cancer cells. Indeed, using immunohistochemistry to analyze the expression of the protein in formalin-fixed paraffin sections of prostate and breast tumor specimens, An et al. discloses the level of the protein is increased in the prostate cancer glandular epithelial cells and breast cancer ductal epithelial cells as compared to the corresponding normal cells; see, e.g., page 7017, the paragraph bridging columns 1 and 2. However, Appellant was reminded that supporting documents published after the filing date sought by Appellant cannot be relied upon to correct the deficiencies of

the specification by supplying the necessary and essential teachings, guidance, and exemplification that the specification lacks. See MPEP § 2164.05(a).

Furthermore, despite teaching its overexpression in breast and prostate cancer cells, An et al. does not teach whether the protein is overexpressed in bladder cancer cells. Again, as evidenced by Chen et al. (of record), for example, one cannot presume that the amount of protein produced in a cell will mirror the amount of mRNA produced. As explained previously, this fact is so universally accepted, it is mentioned in a textbook (i.e., Genes VI, 1997) (of record).

As explained in the preceding Office action, if the protein is expressed at the surface of cells, and the inhibitor is an antibody, unless the cancer cells, relative to normal cells of the same tissue type, more abundantly express the protein, the antibody will not selectively target cancer cells, but will also undesirably target normal cells. One skilled in the art could therefore not use the claimed invention without first performing an undue amount of additional experimentation to determine if the protein encoded by the polynucleotides of SEQ ID NO: 83 and SEQ ID NO: 85 is over-expressed in bladder cancer cells, compared to normal cells of the same tissue type.

In conclusion, although Appellant's arguments traversing this ground of rejection was carefully considered, upon equally careful consideration of the factors used to determine whether undue experimentation is required, in accordance with the Federal Circuit decision of *In re Wands*, 858 F.2d at 737, 8 USPQ2d at 1404 (Fed. Cir. 1988), there is a preponderance of factual evidence of record indicating that the amount of guidance, direction, and exemplification disclosed in the specification would be

insufficient to have enabled the skilled artisan to use the claimed invention at the time the application was filed without undue and/or unreasonable experimentation.

(10) Response to Argument

A. At pages 4-8 of the Brief Appellant has traversed the propriety of the rejection of the claims under 35 U.S.C. § 112, first paragraph, as failing to comply with the written description requirement.

Appellant has argued the claims are directed to "agents" that bind to certain peptides and/or polypeptides (Brief, page 6, paragraph 2) and, because the specification describes an antibody that binds to UC 28 (Brief, page 6, paragraph 3), the specification complies with the written description requirement (Brief, page 6, paragraph 3).

In response, as explained in the Office actions mailed November 22, 2004, and November 10, 2005, the claims are directed to a genus of "agents", which bind to peptide or polypeptide encoded by the nucleotide sequences of SEQ ID NO: 83 or SEQ ID NO: 85, or fragments thereof. Although the genus of "agents" to which the claims are directed includes antibodies, the genus includes other structurally and/or functionally disparate molecules, such as peptides and small molecules, which have no structurally relationship to such an antibodies, but rather only share only a common ability to bind to a peptide or polypeptide encoded by one of the recited nucleotide sequences or a fragment thereof.

In deciding *The Reagents of the University of California v. Eli Lilly*, 43 USPQ2d 1398 (CAFC 1997), the Federal Circuit held that a generic statement that defines a genus of nucleic acids *by only their functional activity* does not provide an adequate written description of the genus. By analogy, a generic statement that defines a genus of "agents" by only their common ability to bind to a peptide or polypeptide encoded by a nucleic acid molecule or fragment thereof does not serve to adequately describe the genus as whole. The Court indicated that while applicants are not required to disclose every species encompassed by a genus, the description of a genus is achieved by the recitation of a precise definition of a representative number of members of the genus, such as by reciting the structure, formula, chemical name, or physical properties of those members, rather than by merely reciting a wish for, or even a plan for obtaining a genus of molecules having a particular functional property. The recitation of a functional property alone, which must be shared by the members of the genus, is merely descriptive of what the members of genus must be capable of doing, not of the substance and structure of the members.

Notably, as also explained previously, although *Lilly* related to claims drawn to genetic material, the statute applies to all types of inventions. "Regardless whether a compound is claimed *per se* or a method is claimed that entails the use of the compound, the inventor cannot lay claim to the subject matter unless he can provide a description of the compound sufficient to distinguish infringing compounds from non-infringing compounds, or infringing methods from non-infringing methods". *University of Rochester v. G.D. Searle Co.*, 69 USPQ2d 1886 1894 (CAFC 2004). The claimed

method depends upon finding an "agent" that has the ability to bind to a peptide or polypeptide encoded by SEQ ID NO: 83 or a fragment thereof, or a peptide or polypeptide encoded by SEQ ID NO: 85 or a fragment thereof to achieve therapeutic effect in treating breast cancer, bladder cancer, or prostate cancer using the claimed process; without such an "agent", it is impossible to practice the invention.

Again, although the specification describes an antibody that binds to UC 28, a protein Appellant has stated in encoded by the nucleotide sequence of SEQ ID NO: 3, SEQ ID NO: 83, and SEQ ID NO: 85, an antibody is not representative of the genus of agents, as a whole, to which the claims are directed. The disclosed antibody shares no disclosed or apparent particularly identifying (i.e., substantial) structural feature with other members of this genus of structurally disparate "agents"; and more pointedly, the antibody shares no such common structural feature that correlates with the shared ability of each of the members of the genus to bind to any peptide or polypeptide that is encoded by the polynucleotide sequence set forth as SEQ ID NO: 83 or a fragment thereof, or as SEQ ID NO: 85 or a fragment thereof. Therefore, the antibody is not representative of the genus of "agents"; and moreover, even given benefit of Appellant's disclosure of the claimed invention, the skilled artisan could not immediately envision, recognize or distinguish at least a substantial number of the members of the genus. As such, contrary to Appellant's contention, the specification would not reasonably convey to the skilled artisan that Appellant had possession of the claimed invention at the time the application was filed, so as to comply with the written description requirement set forth under 35 U.S.C. § 112, first paragraph.

Additionally, though the claims may find literal support in the specification, as explained by the Federal Circuit, even *in ipsis verbis* support does not *per se* establish compliance with the written description requirement:

> Even if a claim is supported by the specification, the language of the specification, to the extent possible, must describe the claimed invention so that one skilled in the art can recognize what is claimed. The appearance of mere indistinct words in a specification or a claim, even an original claim, does not necessarily satisfy that requirement. The disclosure must allow one skilled in the art to visualize or recognize the identity of the subject matter purportedly described. *Eli Lilly*, 119 F.3d at 1568, 43 USPQ2d at 1406.

Regents of the University of California v. Eli Lilly & Co., 119 F.3d 1559, 43 USPQ2d 1398 (Fed. Cir. 1997). *See also: University of Rochester v. G.D. Searle & Co.*, 69 USPQ2d 1886 1892 (CA FC 2004).

In this instance, as explained in the preceding Office actions, the specification discloses that the genus of "agents" to which the claims are directed includes, for example, specific "inhibitors" of the polypeptide encoded by the nucleic acid sequences of SEQ ID NO: 83 or SEQ ID NO: 85. Yet, as also explained previously, although the specification describes the polynucleotides of SEQ ID NO: 83 and SEQ ID NO: 85 as novel (page 111, Table 3), <u>it does not disclose the specific activities or functions of the polypeptides encoded by these nucleotide sequences</u>, which are inhibited by members of the genus of "agents" to which the claims are directed. Consequently, the skilled artisan could not envision such agents, *which are inhibitors of an activity or function that has not been described*, nor could the skilled artisan distinguish a compound capable of inhibiting the activity or function in the absence of such a description. For this reason,

though the claims may find literal support in the specification, the disclosure would not reasonably convey to the skilled artisan that Appellant had possession of the claimed invention at the time the application was filed.

"[G]eneralized language may not suffice if it does not convey the detailed identity of an invention." *University of Rochester v. G.D. Searle Co.*, 69 USPQ2d 1886 1892 (CAFC 2004). In this instance, there is no language that adequately describes "agents" that bind to a peptide or polypeptide encoded by one of the recited nucleotide sequence or a fragment thereof to inhibit its function, which can thereby be used to achieve the claimed therapeutic effect. Again, a description of what a material does, rather than of what it is, does not suffice to describe the claimed invention.

The specification discloses the genus of "agents" includes antibodies and "other inhibitors" (page 18, lines 18-20), which are thereafter not described in any additional detail. While the artisan could potentially make one or more of the polypeptides that is encoded by SEQ ID NO: 83 or a fragment thereof, or by SEQ ID NO: 85 or a fragment thereof, how might one immediately identify a molecule that is capable of binding to the polypeptide, the structure of which has not been described and/or has yet to be determined?

Perhaps the artisan *could* screen candidate molecules (e.g., peptides and small molecules) to identify those that bind the polypeptide, but as previously noted the written description provision of 35 U.S.C § 112 is severable from its enablement provision; and adequate written description requires more than a mere statement that it is part of the invention and reference to a potential method for isolating it.

> The purpose of the "written description" requirement is broader than to merely explain how to "make and use"; the applicant must convey with reasonable clarity to those skilled in the art that, as of the filing date sought, he or she was in possession *of the invention.* The invention is, for purposes of the "written description" inquiry, *whatever is now claimed.*

Vas-Cath, Inc. v. Mahurkar, 935 F.2d 1555, 1563-64, 19 USPQ2d 1111, 1117 (CAFC 1991). See *Fiers v. Revel*, 25 USPQ2d 1601, 1606 (CAFC 1993); *Amgen Inc. v. Chugai Pharmaceutical Co. Ltd.,* 18 USPQ2d 1016 (CAFC 1991); *University of Rochester v. G.D. Searle Co.*, 69 USPQ2d 1886 1892 (CAFC 2004).

Furthermore, perhaps the artisan could use the polypeptide encoded by the recited nucleotide sequences or fragments thereof as an immunogen to produce antibodies that bind to the polypeptide, but how might one immediately identify an antibody that is capable of inhibiting the activity of the polypeptide, which has not been described and/or has yet to be determined?

Without knowledge of the specific activity of the peptide or polypeptide that is necessarily inhibited by the "agent", whether that agent is an antibody or some other nondescript molecule capable of binding the peptide or polypeptide, even the skilled artisan could not immediately identify or distinguish members of the genus of "agents" that might be used to practice the claimed invention to achieve the claimed therapeutic effect in a patient diagnosed with cancer.

At page 6 of the Brief, Appellant's have argued that the disclosure of a first generation polyclonal antiserum produced in rabbits using a KLH-conjugated peptide having the amino acid sequence set forth as SEQ ID NO: 56 should satisfy the written

description requirement; but where is there disclosure of any factual evidence that this antibody binds to the extracellular domain of a peptide or polypeptide encoded by the nucleotide sequence set forth as SEQ ID NO: 83 or SEQ ID NO: 85, or a fragment thereof? Moreover, where is there disclosure of any factual evidence that this polyclonal antibody inhibits a specific activity or function of such a peptide or polypeptide? Finally, absent such disclosures, why would the specification, as filed, reasonably convey, as required under the provisions of the applied statute, that Appellant had possession of the genus to which the claims are directed, or even a representative species of such "agents", which when administered to a patient suffering from breast, bladder or prostate cancer is capable of yielding therapeutic effect?

It is submitted that, at best, the specification might only fairly convey Appellant's possession of the claimed invention in which the "agent" is limited to an immunoconjugate comprising an antibody that binds the protein, which is adjoined to either a radionuclide or chemotherapeutic agent. It would not, however, reasonably convey possession of the claimed invention in which the "agent" is any of the structurally and/or functionally varying molecules that are, or include, specific "inhibitors" of the polypeptide encoded by the nucleic acid sequences of SEQ ID NO: 83 or SEQ ID NO: 85, such as *naked* antibodies that bind the polypeptide and inhibits its specific activity or function, but which are not conjugated to a cytotoxic or cytostatic moiety.

On this note, it is again noted that the Federal Circuit recently decided that the description of a fully characterized molecular target of an antibody is sufficient to adequately describe an antibody that binds that target. <u>See</u> *Noelle v. Lederman*, 69

USPQ2d 1508 (CA FC 2004). However, the same court decided that each case involving the issue of written description, "must be decided on its own facts. Thus, the precedential value of cases in this area is extremely limited." *Vas-Cath*, 935 F.2d at 1562 (quoting *In re Driscoll*, 562 F.2d 1245, 1250 (C.C.P.A. 1977)).

Following the example set by the Federal Circuit in deciding *Noelle v. Lederman*, then, were the claims directed to an antibody that binds a well-characterized antigen, the written description would be met. However, the claims are not solely directed to an antibody that binds a well-characterized molecular target. Rather the instant claims are directed to a genus of structurally and/or functionally disparate "agents" that commonly bind to any peptide or polypeptide that is encoded by SEQ ID NO: 83 or a fragment thereof or a peptide or polypeptide that is encoded by SEQ ID NO: 85 or a fragment thereof, which includes a naked antibody that binds to the peptide or polypeptide and inhibits its activity or function, so as to be therapeutically effective; and yet, as noted above, the specification fails to describe the activity or function of the polypeptide. Moreover, the specification fails to describe an antibody that binds the peptide or polypeptide to specifically inhibit its activity or function; and it fails to describe an antibody that is not conjugated to a radionuclide or chemotherapeutic agent, which would reasonably be expected to inhibit the growth of breast, bladder or prostate cancer cells expressing the peptide or polypeptide at their surfaces.

The specification further fails to describe the "epitope" of the peptide or polypeptide to which such an inhibitory antibody must bind.

Yet, there is factual evidence that the detailed description of an antigen, as opposed to the detailed description of an epitope of an antigen, should not always be regarded as sufficient to describe the antibody that binds that antigen, particularly in instances where binding of the antibody modulates the activity of the antigen. For example, Stancoviski et al. *(Proceedings of the National Academy of Science USA.* 1991; **88**: 8691-8695) characterized the binding effects upon the growth of tumor cells of different antibodies, each of which bind different epitopes of the extracellular domain of a tumor-associated antigen related to EGFR, namely ErbB2; see entire document (e.g., the abstract). Stancovski et al. teaches some anti-ErbB2 antibodies inhibited tumor cell growth, but others actually accelerated their growth (page 8693, column 1). By way of explanation, Jiang et al. (*J. Biol. Chem.* 2005 Feb 11; **280** (6): 4656-4662) teaches that it is well known that different biological effects are associated with epitope specificity of the antibodies; see entire document, particularly page 4656, column 2.

Accordingly, the mere generalized description of antibodies that bind a well-characterized antigen, as opposed to a well-characterized epitope of an antigen, cannot always suffice to describe adequately antibodies that have, for example, an inhibitory or therapeutic effect, because the skilled artisan could not immediately envision, recognize, or distinguish those antibodies that bind an antigen on tumor cells and inhibit the growth of those tumor cells from antibodies that bind the antigen but lack therapeutic effect (e.g., promote the growth of tumor cells).

Accordingly, it is believed that each and every argument set forth in the Brief at pages 4-8 traversing the propriety of the written description rejection has been

addressed. Despite careful consideration of the merits of those arguments, it is submitted that the claims are properly rejected on this ground.

B. At pages 8-14 of the Brief Appellant has traversed the propriety of the rejection of the claims under 35 U.S.C. § 112, first paragraph, as failing to comply with the enablement requirement.

At page 9 of the Brief Appellant has argued that the "agent" to which the claims are directed need not inhibit the specific activity or function of the peptide or polypeptide encoded by the nucleotide sequence of SEQ ID NO: 83 or SEQ ID NO: 85, or a fragment thereof, to prove suitable for use in practicing the claimed invention to achieve the claimed therapeutic effect. Moreover, Appellant has remarked the "agent" need only bind the peptide or polypeptide.

In response, if the "agent" (e.g., an antibody, a peptide, a small molecule) binds the peptide or polypeptide, but does not inhibit its specific activity or function, why might the skilled artisan expect the agent to be capable of yielding therapeutic effect when administered to a patient suffering from breast, bladder or prostate cancer?.

As explained in the preceding Office actions and again in the paragraphs above, unless the agent is conjugated to a cytotoxic or cytostatic moiety it would not be expected to have therapeutic effect, *unless it inhibits the activity or function of the protein to which it binds*, and then in either event only if the protein is expressed at the surface of the targeted cancer cells in the patient.

It is thus for this very reason that it has been submitted that, at best, the specification might only fairly convey Appellant's possession of the claimed invention in which the "agent" is limited to an immunoconjugate comprising an antibody that binds the protein, which is adjoined to either a radionuclide or chemotherapeutic agent. It would not, however, reasonably convey possession of the claimed invention in which the "agent" is any of the structurally and/or functionally varying molecules that are, or include, specific "inhibitors" of the polypeptide encoded by the nucleic acid sequences of SEQ ID NO: 83 or SEQ ID NO: 85, such as *naked* antibodies that bind the polypeptide and inhibits its specific activity or function, but which are not conjugated to a cytotoxic or cytostatic moiety.

So then with regard to the issue of enablement, it is aptly noted that the specification would, at best, only reasonably enable the skilled artisan to practice the claimed invention in which the "agent" is an antibody that specifically binds to the polypeptide encoded by the nucleic acid sequences of SEQ ID NO: 83 or SEQ ID NO: 85, <u>provided that the polypeptide is expressed at the surface of breast, bladder or prostate cancer cells</u>, and only then when the antibody is conjugated to a cytotoxin, for example, which is known to inhibit the growth of the targeted cancer cells.

However, the specification does not establish whether the protein encoded by the nucleic acid sequence of SEQ ID NO: 83 or SEQ ID NO: 85 is expressed at the cell surface by breast, bladder and prostate cancer cells; and as discussed in detail below, there is factual evidence of record indicating just the opposite, i.e., the protein may not

be expressed at the cell surface, so as to be accessible to an antibody administered to a patient suffering from cancer.

As for any "agent", which is not an antibody or antigen binding fragment thereof, such as a peptide or small molecule, the amount of guidance and direction set forth in the specification would not be sufficient to reasonably enable the skilled artisan to make the "agent" that might be suitable for use in practicing the claimed invention to achieve therapeutic effect in patients suffering from breast, bladder, or prostate cancer. While it has become routine to make a monoclonal antibody that specifically binds to a protein (e.g., the peptide or polypeptide encoded by SEQ ID NO: 83 or SEQ ID NO: 85, or a fragment thereof), the production of other ligands (e.g., small molecules), which can be used to selectively target cancer cells expressing the protein by virtue of their ability to bind to the protein, is far less conventional. In this instance, in fact, one cannot even know whether such a molecule exists or can be synthesized; there may well not be a small molecule, for example, capable of specifically binding to the peptide or polypeptide encoded by the recited nucleic acid sequences. As such, the production of other types of "agents", which are not antibodies or antigen binding fragments thereof, falls well within the realm of what is expected to require undue and/or unreasonable experimentation, so as to elaborate upon mere conception, as is disclosed in the application, to develop a fully enabled and effective process for treating cancer.

Appellant is therefore reminded that reasonable correlation must exist between the scope of the claims and scope of enablement set forth.

In deciding *In re Fisher*, 166 USPQ 18, 24 (CCPA 1970), the Court indicated the more unpredictable an area is, the more specific enablement is necessary in order to satisfy the statute. "Tossing out the mere germ of an idea does not constitute enabling disclosure. While every aspect of a generic claim certainly need not have been carried out by an inventor, or exemplified in the specification, reasonable detail must be provided in order to enable members of the public to understand and carry out the invention." *Genentech Inc. v. Novo Nordisk A/S*, 42 USPQ2d 1001, 1005 (CA FC 1997).

The overly broad scope of the claims would merely serve as an invitation to one skilled in the art to identify peptides, small molecules, or other ligands having the ability to bind to a peptide or polypeptide encoded by SEQ ID NO: 83 or SEQ ID NO: 85, which may be suitably used as the "agent" in the practice of the claimed process for treating cancer; yet, defining a substance by its principal biological activity amounts to an alleged conception having no more specificity than that of a wish to know the identity of any material with that biological property. See *Colbert v. Lofdahl*, 21 USPQ2d 1068, 1071 (BPAI 1991).

At page 9 of the Brief Appellant has again remarked that the specification teaches a polyclonal antiserum that was produced using a peptide consisting of the amino acid sequence of SEQ ID NO: 56, which allegedly binds to a polypeptide (i.e., "UC 28") encoded by a nucleotide sequence of the present invention, arguing that as long as the specification discloses at least one method for making and using the

claimed invention that bears a reasonable correlation to the entire scope of the claim, the enablement requirement has been met.

In response, it is submitted that the disclosure of the polyclonal antiserum is not reasonably commensurate in breadth with the scope of the instant claims directed to a process for treating breast, bladder, or prostate cancer in a patient by administering to the patient an "agent" that binds to a peptide or polypeptide encoded by the nucleotide sequence of SEQ ID NO: 83 or a fragment thereof or a peptide or polypeptide encoded by the nucleotide sequence of SEQ ID NO: 85. The specification does not actually demonstrate by the disclosure of factual evidence that this particular preparation of antibodies specifically binds to a peptide or polypeptide encoded by the nucleotide sequence of SEQ ID NO: 83 or SEQ ID NO: 85, or to a polypeptide designated "UC 28". Nonetheless, while it might be reasonably presumed the disclosed polyclonal antibody binds to "UC 28", as Appellant has alleged, it would not be so reasonably presumed that the antibody could be used as the "agent" in practicing the claimed invention to achieve the claimed therapeutic effect in patients suffering from breast, bladder or prostate cancer. Polyclonal antibodies are generally recognized as lacking the requisite specificity of the monoclonal antibody, binding multiple different epitopes of an antigen and any other antigens sharing such epitopes, and are therefore seldom, if ever used effectively to treat cancer. Additionally, as evidenced by the teachings of Stancoviski et al. (*supra*) and Jiang et al. (*supra*), it is well known that different biological effects are associated with epitope specificity of the antibodies. Stancovski et al., for example, teaches some antibodies inhibited tumor cell growth, but others actually accelerated

their growth, despite the fact that all of the antibodies bound to the same protein. It follows that the effects of contacting a protein, such as "UC 28" with a polyclonal antibody that binds multiple different epitopes of the protein may vary widely, perhaps accelerating the growth of cancer cells expressing the protein, or inhibiting their growth, or maybe having no effect whatsoever, if for example the different epitope specific effects are mutually nullified upon binding to multiple different species of antibody.

At page 10 of the Brief Appellant has argued that effective *targeting* of cancer cells with a binding agent does not require prior determination of the function or activity of the polypeptide to which the agent binds. In response, the claims are not directed to a method for targeting cancer cells; rather the claims are directed to a process for treating breast, bladder or prostate cancer. It is not merely sufficient to *target* cancer cells with an agent that binds a polypeptide expressed by the cancer cells to *treat* the cancer. If the targeting agent is not conjugated or attachable *in vivo* to a therapeutic agent (e.g., a cytotoxin or anticancer drug), it is not expected to affect the growth or survival of the cells to which it binds, unless the targeting agent itself is capable of altering the function or activity of the protein, so as to adversely affect the growth or survival of the cells. So, while effective *targeting* of cancer cells with a binding agent does not require prior determination of the function or activity of the polypeptide to which the agent binds, if not conjugated or attachable *in vivo* to a therapeutic agent, effective treatment of cancer does.

However, if the polypeptide to which the antibody binds is not expressed at the surface of the cancer cells, so as to be accessible to the antibody, it will not matter one

way or the other if the antibody is or is not conjugated or attachable *in vivo* to a therapeutic agent. The antibody that binds a protein cannot selectively target cancer cells expressing the protein, unless the protein is expressed at the surface of the cells. As noted above, while the specification teaches the production of a polyclonal antibody, its use has not been exemplified to, for example, localize the protein to which it binds to the surface of breast, bladder or prostate cancer cells; and more pointedly the specification provides no disclosure of factual evidence teaching or reasonably suggesting that the peptide(s) or polypeptide(s) encoded by the nucleotide sequences of SEQ ID NO: 83 and SEQ ID NO: 85, as expressed in such cancer cells, is displayed at the surface of those cells to permit contact by an antibody, such as the disclosed polyclonal antibody.

At page 10 of the Brief Appellant has argued to the contrary that the specification teaches the peptide or polypeptide encoded by the nucleotide sequence of SEQ ID NO: 83 or SEQ ID NO: 85 was found *on the cell membrane*, presumably implying that the peptide or polypeptide is displayed at the surface of those cells to permit contact by an antibody, such as the disclosed polyclonal antibody.

The disclosure to which Appellant has specifically referred as teaching that the peptide or polypeptide is accessible to an antibody administered to the patient is found at page 117, lines 10-14, which reads:

> The synthetic peptide was conjugated to KLH by standard techniques and injected into two rabbits, with bleeding started at ten weeks. The antibody was peptide affinity purified and then tested in prostate cancer cell lines,

and breast and prostate cancer tissue, confirming the localization of the UC 28 protein to epithelial cells, mainly on the cell membrane.

Therefore, in response to Appellant's argument it is noted that the specification teaches the disclosed polyclonal antibody binds to a protein that localized on the cell membrane of epithelial cells; it does not, however, teach or establish as fact that the protein to which the antibody binds is expressed at the surface of the cells, so as to permit an "agent" (e.g., an antibody) administered to the patient afflicted with breast, bladder or prostate cancer to bind selectively to those cells, so as to cause therapeutic effect.

Then, as explained in the preceding Office actions, it is believed that An et al. (*Cancer Research* 2000; **60**: 7014-7020) provides factual evidence that the protein, which is designated therein as UROC28, is <u>not expressed at the surface of cells</u>. An et al. teaches immunohistochemical analyses of glandular epithelial cells of prostate and breast cancers revealed <u>the protein localizes in the nucleus and cytoplasm</u>; see entire document, particularly page 7018, Figure 5.

Additionally, An et al. discloses the presence of the protein in human serum specimens acquired from patients diagnosed with prostate cancer (see, e.g., page 7018, figure 6). Thus, it would appear that the protein to which the polyclonal antibody binds is *secreted* by prostate cancer cells, rather than retained by the cells as a transmembrane protein having an extracellular domain or as external surface associated protein.

Taken all together, the disclosure by An et al. suggests that, rather than expressed at the surface of cells of epithelial origin, the protein is either nuclear or cytoplasmic, or both, and can be secreted. Again, if the protein is not expressed at the surface of targeted cancer cells, the "agent" (e.g., an antibody or other molecule that binds directly to the protein) cannot be used. The "agent" cannot bind the protein, which is expressed within the cell, and would not selectively bind to cells expressing the protein, which is secreted into the blood or lymphatic system, for example, of the patient.

At page 10 of the Brief Appellant's have cited a declaration by Dr. Veltri, which was made of record during the prosecution of a copending, related application (i.e., U.S. Patent Application No. 09/966,762). A copy of this declaration was submitted in response to the Office action mailed April 21, 2005. Appellant has remarked that this declaration states that UC 28 is expressed on the membrane of cancer cells and arguing that therefore the skilled artisan would expect the "agent", which binds to a peptide(s) or polypeptide(s) encoded by SEQ ID NO: 83 or SEQ ID NO: 85, to target cancer cells that overexpress these proteins.

Responding to this argument, the preceding Office action explains that while the merit of the declaration was carefully considered to the extent that it was believed applicable to the issues raised herein, it was not found persuasive to remedy the issues at hand, so as to overcome the stated ground of rejection.

In the declaration, Dr. Veltri has suggested that An et al. (*supra*) does not teach the protein was *not* expressed as an extracellular, plasma membrane-associated, or

trans-membrane protein, only that it was *primarily* localized in the cytoplasm (i.e., the inside of the cell) and more particularly, to at least some extent, in the nucleus; see item #7 at page 3. Agreeably, An et al. does not teach that the protein, or a portion thereof, was *not* exposed at the cell's extracellular surface; but, if it was, its presence or level of expression at the extracellular surface must not have been remarkable.

The declaration states, "the present specification discloses [...] a significant of UC28 localizes to the cell membrane" (item #9 at page 3). There is, however, no factual evidence attached to the declaration to support this assertion. As explained in the preceding Office actions, while the instant specification teaches the protein has been localized to epithelial cells, "mainly on the cell membrane" (page 117, lines 10-14), the specification <u>does not actually teach whether the protein is expressed at the surface of the cells</u>. Given the methodology and resolution of the microscopy used by An et al. it is submitted that it would not be possible to reasonably conclude that the protein is exposed at the surface of the cell; if such, methodology was used by Appellant to determine that the protein is "mainly on the cell membrane", it is further submitted that, using such methodology, one could not reliably distinguish a protein that is localized on the inside surface of the plasma membrane from a protein that is localized in the cytoplasm, or a protein that is transmembrane protein. **Because the tissue sections used in the process are fixed and permeabilized, the antibody is capable of binding antigens both inside and out.** Furthermore, the antibody that was used was a *polyclonal* antibody, so it recognizes many different antigenic determinants on the protein, <u>not just antigenic determinants present on a putative extracellular domain</u>;

therefore, at the resolution used, it would not be possible to determine with any degree of certainty whether the protein is exposed at the surface of living cells.

The specification discloses that the protein has been localized to epithelial cells, "mainly on the cell membrane" (page 117, lines 10-14), but as evidenced by Maddala et al. (*Exp. Cell Res.* 2005; **306**: 203-215), for example, not all proteins that appear localized "mainly on the cell membrane" are transmembrane proteins comprising an extracellular domain that is accessible to an antibody at the outside surface of the cell; see entire document (e.g., the abstract). Maddala et al. teaches localization of α-crystallin, an intracellular protein, to the leading edges of the plasma membrane of lens epithelial cells; see, e.g., the abstract. This protein associates with the plasma membrane, but it is not a transmembrane protein. Using the methodology exemplified by An et al., how might one distinguish a protein, such as α-crystallin from a transmembrane protein, given that both proteins would appear to localize to the plasma membrane? At the resolution used by An et al., it is submitted that such a distinction could not be made with reasonable certainty. The specification provides no disclosure of factual evidence reasonably suggesting that the protein encoded by nucleotide sequences SEQ ID NO: 83 or SEQ ID NO: 85 is a transmembrane protein, as opposed to an intracellular protein that associates with the plasma membrane. The declaration asserts that the protein is accessible to an antibody at the surface of prostate, bladder and breast cancer cells, but provides no showing of factual evidence to support the assertion. An et al., on the other hand, suggests the protein is not a transmembrane protein, but instead a soluble protein localized primarily to the cytoplasm and nucleus.

The declaration further states the use of conventional confocal fluorescence microscopy limited the ability of An et al. (*supra*) to more specifically characterize the localization of the protein and suggests the specification teaches the use of other methodology that remedies the inadequacy of their earlier methodolgy, so as to have permitted the accurate localization of the protein to outside surface of the plasma membrane. Agreeably, the methodology used by An et al. would not permit one to accurately localize the protein, or a portion thereof, to the outside surface of the cell. As evidenced by, for example, the attached references (i.e., Takizawa et al. (*J. Nippon Med. Sch.* 2004; **71** (5): 306-307) and Maddala et al. (*supra*)), the resolution of the microscopy used by An et al. would not have permitted such a conclusion. However, contrary to the statement by Dr. Veltri, there does not appear to be any disclosure in the instant specification of the use of high-resolution confocal immunofluorescent microscopy to localize the protein. In fact, the words "confocal" and "microscopy" do not appear in the specification. Thus, if any merit of the declaration is extended to the instant application, it is not known to which disclosures in the instant specification Dr. Veltri would have referred as providing remedy to the inadequacy of the methodology used by An et al.

The declaration notes the presence of a *putative* transmembrane domain in the protein and states, "the presence of this putative transmembrane domain indicates that UC28 localized to the cell membrane" (item #6). If the domain is only a *putative* transmembrane domain, it has not yet been determined to *be* a transmembrane domain. The disclosure by An et al. suggests that the protein is *not* present at the

extracellular surface, as if it were, its presence there was unremarkable. An et al. teaches the protein primarily localized to the inside of the cell (i.e., the cytoplasm and the nucleus); the results disclosed by An et al. do not suggest that the protein comprises an extracellular domain, or that at least part of the protein is exposed at the surface of cancer cells.

At page 11 of the Brief Appellant has argued that the observation disclosed by An et al. that the protein is primarily localized in the cytoplasm, as opposed to the surface of the cell, does not distinguish the protein as a protein that is exclusively localized to the cytoplasm, and not to the surface, because, as Appellant has alleged, it is perfectly consistent that the protein be in the cytoplasm but also membrane bound.

In support of this position, Appellant has cited the disclosure by An et al. at page 7017, which reads:

> UROC28 protein was localized primarily in the cytoplasm of prostate and breast cancer glandular epithelial cells (Fig. 5*A-D*). However, distinct nuclear localization was also noted in the prostate cancer glandular epithelia (Fig. 5B).

Contrary to Appellant's argument, this disclosure of An et al. is not interpreted as a suggestion that the protein is expressed at the surface of the cell, so as to be immediately accessible to an "agent", such as antibody that binds to at least an extracellular portion of the protein when administered to the patient. As explained in the paragraphs above, An et al. teaches the protein is localized primarily to the cytoplasm of

the prostate and breast cancer epithelial cells, but apparently it may also be found in the nuclei of prostate cancer epithelial cells.

At page 11 of the Brief Appellant has remarked that Dr. Veltri's statement that the protein comprises a putative transmembrane domain is supported by a disclosure in the abstract of An et al. that bioinformatics suggest the occurrence in the protein of a possible transmembrane domain.

In response, the Office does not disagree with the finding that there is a *putative* transmembrane domain in the protein, which was identified using bioinformatics; nonetheless, as explained in the paragraphs above, An et al. provide no indication that the protein is a transmembrane protein comprising extracellular, transmembrane, and intracellular domains. Instead An et al. teaches the protein was found by immunohistochemical analyses to primarily localize to the cytoplasm and/or the nucleus.

As Appellant has correctly noted at page 12 of the Brief: "[A] specification disclosure which contains a teaching of the manner and process of making and using the invention in terms which correspond in scope to those used in describing and defining the subject matter sought to be patented must be taken as in compliance with the enabling requirement of the first paragraph of § 112 unless there is reason to doubt the objective truth of the statements contained therein which must be relied on for enabling support." *In re Marzocchi*, 439 F.2d 220, 223, 169 USPQ 367, 369 (CCPA 1971).

In this instance, the Office has met its burden, finding factual evidence teaching or reasonably suggesting there is reason to doubt the objective truth of the statements

contained in the specification, which must be relied on for enabling support of the claimed invention. The specification provides no disclosure of factual evidence reasonably suggesting the protein or a portion thereof is accessible at the surface of the cell. To the contrary, An et al. (*supra*) teaches *only* that the protein is expressed and localized to the <u>inside of the cell</u>, in either the cytoplasm or the nucleus, or both; An et al. does not provide any factual evidence supporting Appellant's position that the protein comprises at least a portion that is present at the surface of the cell, so as to be accessible to the "agent" following its administration to the patient. Consequently, the claimed process could not be used without undue and/or unreasonable experimentation to first validate the very premise upon which its claimed effectiveness in treating cancer is based, namely the necessary accessibility of the protein, as it is expressed by breast, bladder, and prostate cancer cells, to an administered "agent" capable of binding to the protein.

At page 12 of the Brief Appellant has asserted the use of "anticancer agents of the invention" would not require undue experimentation since, for example, the "agents" capable of binding a peptide or polypeptide encoded by SEQ ID NO: 83 or SEQ ID NO: 85 may be conjugated to radionuclides or chemotherapeutic drugs, and the use of such is well known in the art.

In response, the prior Office actions clearly stated, provided the protein is expressed at the surface of breast, bladder and prostate cancer cells, the specification would reasonably enable the skilled artisan to practice the claimed invention, wherein the "agent" is an antibody or antigen binding fragment thereof, which is conjugated to a

cytotoxic moiety, such as a chemotherapeutic drug. However, many of the rejected claims are not so limited; and again, there is no disclosure of factual evidence reasonably suggesting the protein is accessible at the surface of the targeted cancer cells.

Even so, there is a preponderance of factual evidence now of record that suggests the amount of guidance, direction, and exemplification disclosed in the specification would not reasonably enable the skilled artisan to use even the inventions of claims 83, 84, 92, or 93, which are directed to processes that comprise administering antibodies conjugated or linked to radionuclides or chemotherapeutic agents.

The use of none of claimed processes of claims 83, 84, 92, and 93 has been exemplified.

The specification discloses the production of a polyclonal antiserum but does not provide a showing that the antibodies are capable of specifically binding to a peptide or polypeptide encoded by the nucleotide sequences of SEQ ID NO: 83 or SEQ ID NO: 85. Moreover, it does not demonstrate the use of the antibodies to selectively target cancer cells expressing the peptide or polypeptide.

Polyclonal antibodies, nevertheless, lack the requisite specificity that monoclonal antibodies possess, and are therefore seldom if ever used effectively to treat cancer.

While the skilled artisan could readily make a monoclonal antibody for use in practicing the claimed process, there is no disclosure of factual evidence that would be viewed as reasonably supporting the assertion that the peptide or polypeptide to which

such an antibody would bind is accessible at the surface of breast, bladder and prostate cancer cells.

An et al. teaches the protein is localized to the inside of the cell, either in the cytoplasm or the nucleus, or both.

An et al. further teaches the protein may be secreted by prostate cancer cells, as it was detected in serum specimens acquired from patients diagnosed with prostate cancer (see, e.g., page 7018, figure 6). During the course of prosecution, Appellant referred to a commentary by Carrol, addressing the importance of findings disclosed by Milowsky et al. (Exhibit A, attached to the response filed April 21, 2005). Milowsky et al. describes a radioactively labeled antibody that specifically binds prostate-specific membrane antigen (PMSA) that is used to treat patients with prostate cancer. Notably, Carrol comments that the antigen to which the antibody binds is an excellent target <u>because it is not secreted</u> like PSA or PAP. Accordingly, Carrol provides factual evidence that the polypeptide encoded by SEQ ID NO: 83 or SEQ ID NO: 5, if secreted by prostate cancer cells, is for that very reason a less desirable target than PMSA.

Furthermore, Carrol emphasizes that the reason that PMSA is an excellent target is that the antibody that recognizes the protein <u>binds tightly to its extracellular domain</u>, as previous monoclonal antibodies bound to an intracellular domain only accessible in already dead or dying cells. Here, as explained above, it is not known whether the protein encoded by SEQ ID NO: 83 or SEQ ID NO: 85 comprises an extracellular domain that might serve as the target of an antibody, which is conjugated to a radionuclide. If the "agent" (e.g., an antibody or other molecule) cannot bind the protein,

it cannot bind the cancer cells; and if those cells secrete the protein, it's binding to the protein will not affect the cells that secreted the protein.

Then, even if the protein were expressed at the surface of the targeted cancer cells, as explained in the prior Office actions, while antibody-targeted therapy can overcome some of the intrinsic shortcomings that reduce the efficacy of agents that are non-selective or non-tumor-specific, there are well known limitations in the art of antibody-targeted therapeutic regimens.

Vitetta et al. (*Cancer Research* 1994; **54**: 5301-5309) teaches: "[D]espite [...] intellectual appeal, the general therapeutic efficacy of tumor-reactive MAbs [monoclonal antibodies] has been disappointing. In particular the results of clinical studies in patients with solid tumors showed little efficacy, except in the setting of minimal disease" (citations omitted) (page 5301, column 1). Vitetta et al. teaches that there are a number of significant limitations in their use as first-line therapy for solid tumors (page 5305, columns 1-2):

> Only 0.001 to 0.1% of injected MAb [monoclonal antibody] will localize to each [gram] of tumor mass. Moreover, MAbs, even at high serum concentrations, cannot gain access to all the cells in solid epithelial tumor. The reasons for this are poor and heterogeneous blood supply, the blood-tumor barrier, and the selective binding of the MAb by the tumor cells closest to the blood supply. In addition, MAbs by themselves probably cannot kill the 10^{10}-10^{12} malignant cells that may be necessary to cure a patient with a disseminated tumor (citations omitted) (page 5305, columns 1-2).

It was further noted in the preceding Office actions that the strategic approach to treating cancer using antibody therapy is analogous to active specific immunotherapy (e.g., vaccination against tumor-associated antigens), at least to the extent that the latter theoretically induces a humoral immune response (i.e., the production of tumor-specific antibody). Antibody therapy can be defined as passive immunization, cancer vaccine therapy as active immunization. Because the efficacy of both approaches depends upon the effectiveness of tumor antigen-specific antibodies to ameliorate or inhibit tumors, both also share the same or corresponding limitations. Bodey et al. (*Anticancer Research* 2000; **20**: 2665-2676) teaches:

> Animal models, albeit highly artificial, have yielded promising results. Clinical trials in humans, however, have been somewhat disappointing. Although general immune activation directed against the target antigens contained with a cancer vaccine has been documented in most cases, reduction in tumor load has not been frequently observed, and tumor progression and metastasis usually ensue, possibly following a slightly extended period of remission. The failure of cancer vaccines to fulfill their promise is due to the very relationship between host and tumor: through a natural selection process the host leads to the selective enrichment of clones of highly aggressive neoplastically transformed cells, which apparently are so dedifferentiated that they no longer express cancer cell specific molecules. Specific activation of the immune system in such cases only leads to lysis of the remaining cells expressing the particular TAAs [tumor associated antigens] in the context of the particular human leukocyte antigen (HLA) subclass and the necessary costimulatory molecules. The most dangerous clones of tumor cells however lack these features and thus the cancer vaccine is of little use.

Thus, as Bodey et al. explains, if a cancer cell does not express the protein that is specifically bound by the antibody at its surface, the use of a pharmaceutical composition comprising such an antibody will not be effective; but, in addition, as Bodey et al. teaches, the use of such a pharmaceutical composition may paradoxically serve to select against tumor cells that express the protein, while promoting the growth of tumor cells that do not express the protein.

It is because of such unpredictability that Gura (*Science.* 1997; **278**: 1041-1042), for example, teaches that researchers are faced with a problem when sifting through potential anticancer agents to find ones promising enough to make human clinical trials worthwhile (abstract). Because of a lack of predictability, Gura discloses that often researchers merely succeed in developing a therapeutic agent that is useful for treating the animal or cell that has been used as a model, but which is ineffective in humans, and indicates that the results acquired during pre-clinical studies are often non-correlative with the results acquired during clinical trials (page 1041, column 2). Gura very succinctly teaches our lack in ability to reliably extrapolate pre-clinical data to accurately predict the outcomes of such treatments in humans is due to the fact that "xenograft tumors don't behave like naturally occurring tumors in humans" (page 1041, column 2). Gura teaches that although researchers had hoped that xenografts would prove to better models for studying cancer in humans and screening candidate therapeutic agents for use in treating patient diagnosed with cancer, "the results of xenograft screening turned out to be not much better than those obtained with the

original models". Gura states that as a result of their efforts, " '[w]e had basically discovered compounds that were good mouse drugs rather than good human drugs' ".

So, despite the accuracy of Appellant's argument beginning at page 12 of the Brief that there are examples of antibodies, which are conjugated or linked to radionuclides or chemotherapeutic drugs, which are well known in the art and which have proven useful in a clinical setting, the successful development and application of effective means of treatment in the art of oncology is nevertheless challenged, if not limited by oft unpredictable and complex nature of biology.

Then, beginning at page 13 of the Brief Appellant has remarked that the specification teaches mRNA molecules encoding UC 28 are overexpressed in breast and bladder cancer cells, and the gene's expression is inducible in prostate cancer cells, arguing that such evidence implicitly indicates that the protein is coordinately overexpressed.

The Office disagrees. As explained in the preceding Office actions, Chen et al. (*Molecular & Cellular Proteomics* 2002; **1**: 304-313), for example, provides factual evidence that one *cannot presume* that the amount of protein produced in a cell will mirror the amount of mRNA produced. So universally accepted is this fact, it is mentioned in a textbook.

Lewin has written: "But having acknowledged that control of gene expression can occur at multiple stages, *and that production of RNA cannot inevitably be equated with production of protein*, it is clear that the overwhelming majority of regulatory events

occur at the initiation of transcription" (italicized for emphasis) (Genes VI, 1997; Ed. Benjamin Lewin; Chapter 29, first page).

Accordingly, it is believed that Appellant's arguments traversing the propriety of the enablement rejection have been addressed.

(11) Related Proceeding(s) Appendix

No decision rendered by a court or the Board is identified by the examiner in the Related Appeals and Interferences section of this examiner's answer.

For the above reasons, it is believed that the rejections should be sustained.

Respectfully submitted,

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A Novel Peptide Isolated from a Phage Display Peptide Library with Trastuzumab Can Mimic Antigen Epitope of HER-2*

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Trastuzumab, a humanized antibody to HER-2, has been shown to be effective in the treatment of breast cancer in which HER-2 overexpression and metastasis occurs. In our search for an effective mimic epitope of HER-2 binding with trastuzumab and to develop HER-2 peptide vaccine, we screened a phage display 12-mer peptide library with trastuzumab as the target. A mimetic peptide (mimotope) H98 (LLGPYELWELSH) that could specifically recognize trastuzumab was isolated. The DNA encoding peptide H98 was cloned and expressed as the fusion protein GST-H98 in *Escherichia coli* BL21. The purified GST-H98 could specifically bind to trastuzumab and block the binding of trastuzumab to HER-2 protein. Moreover, H98 could significantly block the function of trastuzumab inhibiting the growth of cancer cells. Mice that were immunized with GST-H98 made specific antibody to H98 as well as to HER-2. In addition, T-cell proliferation occurred in mice immunized with GST-H98. Although no sequence homology was found between H98 and HER-2, through the use of structure analysis we were able to determine that peptide H98 contributed to a conformational epitope of HER-2. Furthermore, we determined that the last two amino acids at the C terminus, and the third together with the fourth amino acid at the N terminus of peptide H98 are critical to the binding of H98 to trastuzumab. As a result, we conclude that peptide H98 has potential for being developed as a HER-2 vaccine for biotherapy of cancer with HER-2 overexpression.

HER-2 (also known as Neu or ErbB-2) is a member of the epidermal growth factor receptor (also known as HER) family of receptor tyrosine kinase (1). HER receptors are essential mediators of cell proliferation and differentiation in the developing embryo and in adult tissues (2). Overexpression of the HER-2 oncogene is a frequent molecular event in multiple human cancers, including breast, ovarian, gastric, and colorectal carcinomas (3). In patients with breast cancer, HER-2 overexpression is an independent predictor of survival. It is associated with poor prognosis, aggressive disease, resistance to chemotherapy and hormone therapy (4–6). The critical role of HER-2 in epithelial oncogenesis as well as its selective overexpression on malignant tissues makes it an ideal target for immunotherapy (7). Monoclonal antibodies directed to HER-2 induce phenotypic changes in tumor cells including down-modulation of the HER2 receptor, inhibition of tumor cells growth, reversion of cytokine resistance, restoration of E-cadherin expression levels, and reduction of vascular endothelial growth factor production (8). The humanized anti-HER-2/neu antibody trastuzumab (Herceptin; Genentech, Inc., South San Francisco, CA) has been proven to be effective in clinical trials in patients with HER-2-associated metastatic breast cancer (9), and has antitumor activities as both a single agent and in combination with chemotherapy (10). The molecular mechanisms underlying these growth inhibitory effects are not entirely clear. There is some evidence that antibodies to HER-2 may antagonize the constitutive growth signaling properties of the HER-2 system, enlist immune cells to attack the tumor target, and augment chemotherapy-induced cytotoxicity (8).

The clinical application of trastuzumab has shown efficacy. However, several important drawbacks (generation of anti-idiotypic antibodies, inadequate tissue distribution, levels necessitating multiple infusions and hence the associated cost) limit the usefulness of passive immunotherapy protocols (11). On the other hand, vaccination strategy is an alternative option that can elicit endogenous tumor inhibitory antibodies, stimulate immunologic memory, and accordingly provide long-term benefits to patients at lower costs. The use of a peptide or part of a protein rather than a whole protein as vaccines may circumvent tolerance against self-protein HER-2 and induce cross-reactive immunity.

Although many anti-HER-2 antibodies inhibit the proliferation of cancer cells, some actively stimulate cancer growth. It is well known that different biological effects are associated with the epitope specificity of the antibodies. Isolation of epitopes recognized by trastuzumab, an anti-tumor therapeutic monoclonal antibody directed against HER-2, can be useful in the development of peptide-based vaccines that are capable of stimulating an immune response directed to tumors. The screening of phage display libraries is a powerful technique that has been used in epitope or ligand mapping studies to define peptides that bind to a given antibody or receptor molecule (12–15). In this study, we used a phage display 12-mer peptide library to identify peptides that bind to trastuzumab, and tested their immune and biological activity. The screening of a 12-mer peptide library displayed on M13 filamentous phages led to the

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The atomic coordinates and structure factors (code 1Y2N) have been deposited in the Protein Data Bank, Research Collaboratory for Structural Bioinformatics, Rutgers University, New Brunswick, NJ (http://www.rcsb.org/).

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isolation of peptide H98 (LLGPYELWELSH). Further investigation showed that peptide H98, mimicking the binding epitope on HER-2 for trastuzumab, specifically blocked the binding of trastuzumab to HER-2 and elicited an anti-HER-2 antibody response as well as a cellular immune response in mice. Although no sequence homology was found between the mimotope and HER-2, given the known crystal structure of the binding domain of HER-2 and trastuzumab, we found that peptide H98 contributed to a conformational epitope of HER-2. Moreover, the last two amino acids at the C terminus, and the third together with the fourth amino acid at the N terminus of H98, were critical for the binding of H98 to trastuzumab. In addition, MutN1, a mutant of H98, enhanced the binding of trastuzumab to pro-peptide. The approach of using phage display peptide libraries to reveal conformational epitopes may play an important role in research of the immune activity of the HER-2 mimotopes. And the isolated peptide could become a candidate as a vaccine for the treatment of HER-2-associated cancers.

EXPERIMENTAL PROCEDURES

Phage Library, Bacteria, Antibodies, Expression Vector, and Cells—The Ph.D.-12 Phage Display Peptide Library Kit E8110S was purchased from New England Biolabs (Beverly, MA). The library contained 1.5×10^{13} pfu[1]/ml with a complexity of 2.7×10^9 transformants. The displayed peptides were expressed at the N terminus of the pIII coat protein of the filamentous coliphage M13. The library was stored in Tris-buffered saline, pH 7.5, with 50% glycerol. Phage was propagated in *Escherichia coli* strain ER2738, which was provided with the kit.

Antibody trastuzumab was provided by Breast Cancer Center at Beijing Cancer Hospital. Horseradish peroxidase (HRP)-conjugated anti-M13 antibody and the glutathione *S*-transferase (GST) gene fusion system were purchased from Amersham Biosciences. The dihydrofolate reductase (DHFR) gene fusion system was purchased from Qiagen (Valencia, CA).

The human breast cancer cell line SKBR3 (American Type Culture Collection, Manassas, VA) was cultured in Dulbecco's modified Eagle's medium (Hyclone), supplemented with 10% fetal calf serum. NIH3T3-ErbB2 cell line (provided by Dr. S. L. Sun, Peking University School of Oncology) was cultured in RPMI 1640 medium (Hyclone) supplemented with 10% fetal calf serum.

Bio-panning and Selection of Phage—Human normal IgG and trastuzumab were separately immobilized on protein A-agarose beads (Amersham Biosciences). Phages (1.5×10^{11} pfu) were preabsorbed on beads containing immobilized human normal IgG to remove any phages that were not specifically reactive with trastuzumab. The phages, diluted in Tris-buffered saline containing 0.1% Tween 20, were incubated at 4 °C for 1 h with immobilized trastuzumab. The beads were washed 5 times with 0.1% Tween 20/Tris-buffered saline. Then the phages that bound with trastuzumab and beads complex were amplified by direct infection with *E. coli* ER2738. The amplified phages were purified by precipitation with 20% polyethylene glycol 8000 (PEG8000), 2.5 M NaCl and used in the next cycle. Three rounds of selection were performed. After that, individual plaques were picked up randomly and subjected to analysis by phage enzyme-linked immunosorbent assay (ELISA) and DNA sequencing, following amplification in *E. coli* ER2738.

Phage ELISA—In total, 300 phage clones were tested for reactivity with trastuzumab by ELISA. A single clone was grown for 4.5 h in 1 ml of Luria broth (LB) and shaken at 37 °C. Bacterial cells were spun down, and the supernatant containing phage was added to the wells of a microtiter plate that was coated with 5 μg/ml trastuzumab or control human normal IgG in 0.1 M $NaHCO_3$, pH 9.5, and then blocked with 5% skim milk in Tris-buffered saline. The plate was incubated at room temperature for 1 h and washed 5 times with 0.05% Tween/Tris-buffered saline. Bound phages were detected by incubation with HRP-conjugated anti-M13 antibody (Amersham Biosciences) for 1 h, followed by washing and the addition of a peroxidase substrate (*o*-phenylenediamine, 0.4 mg/ml) in citrate-phosphate buffer, pH 5.0, containing 0.02% (v/v) H_2O_2. The reaction was stopped with 50 μl of 12.5% H_2SO_4. A_{492} was determined by using a microplate reader (Bio-Rad model 550).

DNA Sequencing and Peptide Synthesis—Single-stranded phage DNA was prepared from 26 immunopositive clones by standard techniques as described in the phage display peptide library kit and sequenced by Shenyou Biology Inc. (Shanghai, China). Peptides representing the sequence of the positive clone H98 (LLGPYELWELSH) and an irrelevant control random peptide F56 (WHDPTPWWSWET) were synthesized by Bio-Scientific Inc. (Xian, China).

Expression of Recombinant GST Peptide and DHFR Peptide Fusion Proteins in E. coli—To detect the activity of peptide H98 simply and conveniently, GST-H98 and DHFR-H98 fusion proteins were prepared, respectively. To make the GST-H98 fusion protein, the sense (5′-GATCCTTGTTGGGTCCGTATGAGTTGTGGGAGCTTTCTCATTGAAAGCTTG) and the antisense (5′-TCGACAAGCTTTCAATGAGAAAGCTCCCACAACTCATACGGACCCAACAAG) oligonucleotide fragments encoding the positive clone H98, with stop codon, HindIII site, and sticky ends of BamHI/SalI shown by the underlined nucleotides, were synthesized by Sangon Company (Shanghai, China) and inserted at the BamHI and SalI site of the pGEX-4T-1 vector (Amersham Biosciences). The ligated DNAs were used to transform *E. coli* BL21 cells. DNA of recombinants was identified by digestion with HindIII and EcoRV and then confirmed by DNA sequence analysis. All of these procedures were carried out as described by Sambrook *et al.* (16). Purification of DNA from agarose gels was performed with a QIAquick DNA purification kit (Qiagen, Valencia, CA).

For the preparation of GST-H98 fusion proteins, transformed bacteria were cultured in LB medium with ampicillin selection to an optical density of 0.8 at 600 nm. Next, 0.5 mM isopropyl-1-thio-β-D-galactopyranoside was added and the cultures were further incubated for 5 h at 30 °C. Cells were collected by centrifugation and resuspended in 20 ml of phosphate-buffered saline (PBS) with 1 mM phenylmethanesulfonyl fluoride and 1 mg/ml lysozyme. Cell suspensions were sonicated for 1 min at a 20% pulse. After the lysate was cleared by centrifugation at 12,000 rpm for 10 min at 4 °C, the GST-H98 fusion protein was purified with glutathione beads according to the manufacturer's instructions (Amersham Biosciences).

To obtain DHFR-H98 fusion protein, the sense and antisense oligonucleotide fragments as described above were inserted at the BglII (the same sticky end as BamHI) and SalI sites of the pQE-40 vector (Qiagen). The ligated DNAs were used to transform *E. coli* SG13009 cells. The DHFR-H98 fusion protein was purified according to the manufacturer's instructions (Qiagen).

ELISA—The reactivity of trastuzumab with GST and GST-H98 fusion protein was tested by ELISA. 96-Well microtiter plates were coated with 5 μg/ml GST or GST-H98 fusion protein in 0.1 M $NaHCO_3$, pH 9.5, then blocked with 5% skim milk in PBS. The plates were added to dilutions of trastuzumab and held for 2 h at room temperature. Bound trastuzumab was detected using HRP-conjugated anti-human IgG antibody (Zhong Shan Co., Beijing, China) with 0.4 mg/ml *o*-phenylenediamine as a peroxidase substrate, as described under "Phage ELISA."

Western Blot—The reactivity of trastuzumab with GST fusion protein was also tested by Western blot analysis. Two μg of GST, GST-H98, GST-HER-2, and DHFR-H98 fusion proteins were subjected to 12% SDS-PAGE and transferred to nitrocellulose. The proteins were probed with the anti-GST antibody to detect the expression of the GST fusion protein. They were also probed with either trastuzumab or human IgG antibody. After being washed, the filters were incubated with HRP-conjugated secondary antibody and then developed with an electrochemiluminescence (ECL) system (Amersham Biosciences).

Competitive Inhibition Assay—To test the inhibition of trastuzumab binding to HER-2, we coated 96-well microtiter plates with 5 μg/ml NIH3T3-ErbB2 cell lysate (which was prepared by lysing the cells in lysis buffer containing 1% Triton X-100, 1 mM EDTA, 1 mM phenylmethanesulfonyl fluoride, and 1 μg/ml aprotinin) or GST-H98 fusion protein in 0.1 M $NaHCO_3$, pH 9.5. Next, 0.5 μg/ml trastuzumab was mixed with dilutions of H98, F56, GST, GST-H98, and NIH3T3-ErbB2 cell lysate, and preincubated for 1 h at room temperature, and then transferred to the plates. Bound trastuzumab was detected by standard ELISA as described above. The peptides or proteins were tested at 0, 0.5, 5, 10, and 50 μg/ml. Each dilution was tested in duplicate. The inhibition was calculated by using the following formula: $(A_{\text{Trastuzumab}} - A_{\text{Trastuzumab with protein}})/A_{\text{Trastuzumab}} \times 100\%$. The experiment was repeated three times.

Cell Proliferation Assay with MTT—The human breast cancer cell line SKBR3 overexpressing HER-2 were seeded in 96-well microtiter plates at a density of 1×10^4 cells/well. After incubation overnight at 37 °C, Dulbecco's modified Eagle's medium containing either 1 μg/ml trastuzumab or human normal IgG with or without dilutions of peptides was added to the wells in a final volume of 150 μl. All treatments

[1] The abbreviations used are: pfu, plaque-forming units; LB, Luria broth; ELISA, enzyme-linked immunosorbent assay; HRP, horseradish peroxidase; MTT, thiazolyl blue (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide); DHFR, dihydrofolate reductase.

were performed in triplicate. The plates were incubated at 37 °C for 3 days. Numbers of live cells were measured using a thiazolyl blue (MTT, Sigma) cell proliferation assay by reading A_{492}. The proliferation inhibition rate was calculated by the formula as follow: $(A_{\text{Human normal IgG}} - A_{\text{Trastuzumab+H98/F56}})/A_{\text{Human normal IgG}} \times 100\%$.

Immunization—Six- to 8-week-old BALB/c mice (purchased from the Animal Center of the Chinese Medical Academy) were immunized subcutaneously with 40 μg of GST-H98 or GST alone that was emulsified in complete Freund's adjuvant the first time. Mice were boosted four times with the antigen emulsified in incomplete Freund's adjuvant every 3 weeks. Sera were taken on day 0 (preimmune) and 7 days after the third and fifth immunization.

Antibody level in the sera of immunized mice was determined by ELISA. To test the antipeptide response, 96-well microtiter plates were coated with 5 μg/ml DHFR-H98 fusion protein in 0.1 M $NaHCO_3$, pH 9.5, and then blocked with 5% skim milk in PBS. A 1:1000 dilution of mice sera was added to the plates, which were then held for 2 h at room temperature. Bound antibody was detected by using HRP-conjugated anti-mouse IgG antibody with 0.4 mg/ml *o*-phenylenediamine as the peroxidase substrate. To test the anti-HER-2 response, 96-well microtiter plates were coated with 1×10^4 cells/well of NIH3T3-ErbB2 and then blocked with 5% skim milk in PBS. A 1:100 dilution of mice sera was added to the plates and anti-HER-2 response was assessed by standard techniques as described above.

T Cell Proliferation Assay—Spleen cells from 2 randomly selected BALB/c mice, which were immunized 4 times with GST-H98 or GST-other peptide, were isolated according to the standard techniques on day 7 post-immunization (17). 96-Well microtiter plates were coated with peptide H98 at different concentrations (0, 1, 10, and 100 μg/ml) and then washed twice with PBS. The splenocytes (2×10^5/well) were cultured in the coated 96-well plate and incubated at 37 °C for 3 days. The number of live cells were measured by using the thiazolyl blue (MTT, Sigma) cell proliferation assay by reading A_{492}. The proliferation percentage was calculated by the formula as follows: $(A_{H98} - A_{\text{untreated}})/A_{\text{untreated}} \times 100\%$. Each assay was performed at least in triplicate. The experiment was repeated twice.

Structural Determinants of the Binding Sequence and Optimization of the Amino Acid Sequence—To analyze the minimal requisites for binding activity, a variety of truncation and mutation peptides were made fused with GST (Table I). The corresponding GST-truncate, GST-mutant, and GST-cyclic H98 peptide fusion proteins were expressed and purified as described above. The reactivity of trastuzumab with these GST fusion peptides was tested by ELISA and Western blot.

RESULTS

Phage Selection—To select the positive clones that bind to trastuzumab, a random 12-mer phage display peptide library composed of 1.5×10^{11} independent phage clones was preabsorbed by human serum IgG and then specifically absorbed with trastuzumab. For each biopanning, phages were titrated for pfu in the inputs and outputs to determine the degree of selection. The total number of phages bound to trastuzumab was increased from 8.2×10^4 pfu in the first round to 3.7×10^6 pfu in the third round.

Specificity of Peptides Binding to Trastuzumab and Their Sequences—After 3 rounds of selection, roughly 13.7% (41/300) of the phage clones analyzed exhibited trastuzumab binding activity (data not shown). We sequenced 26 positive phage clones and 25 clones encoded the identical amino acid sequence (LLGPYELWELSH) named H98. Only one different sequence, H23 (HPRPYHHTLPLT), was seen. These two sequences are considerably different and have no obvious similarities in motif.

The binding of trastuzumab to the fusion protein GST-H98 was also demonstrated by ELISA and Western blotting. For this experiment, oligonucleotide encoding the peptide H98 was expressed as a C-terminal extension of the GST protein. The results of the ELISA showed that trastuzumab could specifically bind to GST-H98 but not to GST (Fig. 1). As shown in Fig. 1, trastuzumab reacted with the GST fusion protein containing the H98, whereas no reaction was obtained with GST. The results of Western blotting also suggested that trastuzumab could specifically bind to H98 (Fig. 2).

TABLE I
Truncations and mutants of peptide H98

Peptide H98 truncated at the N (N10 to N6), C (C10 to C6), or both N and C (M8 and M6) terminus, respectively. The mutant amino acids as well as additional cysteines were shown in boldface and underlined (MutN3–4, MutC1–2, MutN1–2, MutN1, cyclic H98).

Peptide	Amino acid sequence
H98	LLGPYELWELSH
N10	LLGPYELWEL
N8	LLGPYELW
N6	LLGPYE
C10	GPYELWELSH
C8	YELWELSH
C6	LWELSH
M8	GPYELWEL
M6	PYELWE
MutN3–4	LL**VA**YELWELSH
MutC1–2	LLGPYELWEL**GA**
MutN1–2	**GA**GPYELWELSH
MutN1	**Q**LGPYELWELSH
Cyclic H98	**C**LLGPYELWELSH**C**

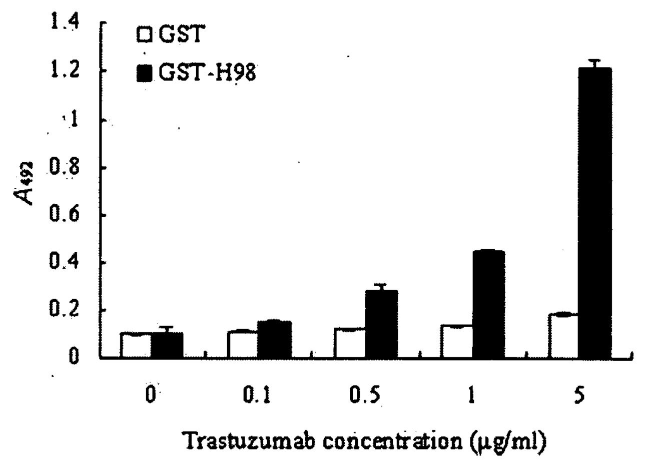

FIG. 1. **Binding of trastuzumab to GST-H98.** Microtiter wells were coated with 5 μg/ml GST-H98 or GST and then blocked with 5% skim milk. Trastuzumab was added to the wells. Bound trastuzumab was detected with HRP-conjugated anti-human IgG antibody and then with the substrate. The values shown are A_{492} and the mean of triplicate samples. S.D. are indicated by *error bars*.

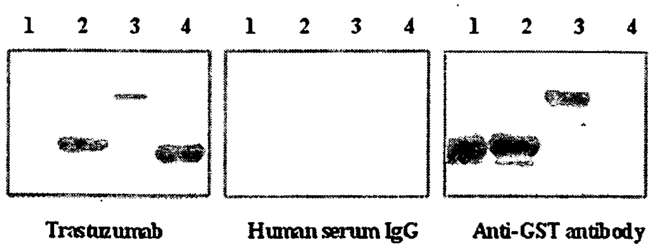

FIG. 2. **Western blot analysis of binding of trastuzumab to peptide.** Different GST fusion proteins were prepared as described under "Experimental Procedures." Equal amounts (2 μg) of proteins were subjected to SDS-PAGE and transferred to nitrocellulose membranes. The proteins were probed with anti-GST antibody (*right panel*) to detect the expression of GST fusion peptides and then probed with either trastuzumab (*left panel*) or human serum IgG (*middle panel*). *Lane 1*, GST; *lane 2*, GST-H98; *lane 3*, GST-HER-2; *lane 4*, DHFR-H98.

GST-H98 and Peptide H98 Inhibition of Trastuzumab Binding to HER-2—The results from the ELISA and Western blot above showed that peptide H98 bound to the variable regions of trastuzumab. However, to demonstrate that H98 resembled the epitope of HER-2, we had to show that H98 blocked binding between trastuzumab and HER-2. To perform these experiments, we used an ELISA in which antibody and inhibitors

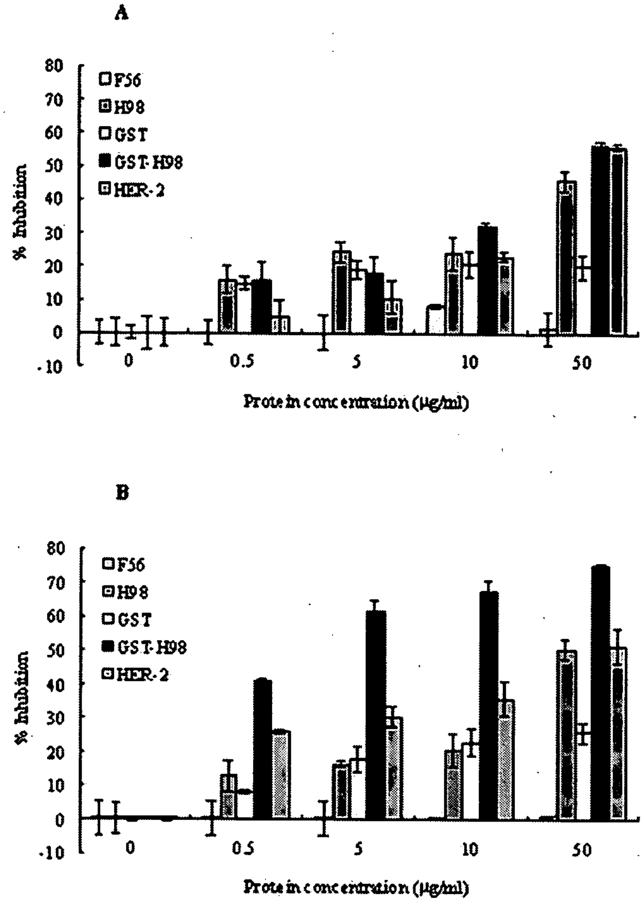

FIG. 3. **Competitive inhibition activity of peptide H98 or GST-H98.** *A*, peptide H98 inhibited trastuzumab from binding to HER-2. Previously, 0.5 μg/ml trastuzumab was mixed with H98, F56, GST, GST-H98, and NIH3T3-ErbB2 cell lysate in the indicated concentrations, preincubated for 1 h, and then transferred to HER-2-coated plates. Bound trastuzumab was detected with HRP-conjugated anti-human IgG antibody and then to the substrate. *B*, HER-2 inhibited trastuzumab from binding to peptide H98. First, 0.5 μg/ml trastuzumab was mixed with H98, F56, GST, GST-H98, and NIH3T3-ErbB2 cell lysate in different concentrations. One hour later, the mixture was added to GST-H98-coated wells. Bound trastuzumab was detected with HRP-conjugated anti-human IgG antibody and then to the substrate. The inhibition rate was calculated according to the following formula: $(A_{\text{Trastuzumab}} - A_{\text{Trastuzumab with Protein}})/A_{\text{Trastuzumab}} \times 100\%$. S.D. are indicated by *error bars*.

(GST-H98 or peptide H98) were preincubated and plated onto the microtiter plates coated with HER-2. This analysis revealed that GST-H98 and peptide H98 inhibited the binding of trastuzumab to HER-2 in a dose-dependent manner, but GST protein and irrelevant peptide F56 did not produce noteworthy inhibition on their interaction (Fig. 3*A*).

HER-2 Inhibiting Trastuzumab Binding to GST-H98—To demonstrate that the trastuzumab that bound to GST-H98 or peptide H98 was specific for HER-2, we used HER-2 to inhibit trastuzumab binding to GST-H98. Trastuzumab was premixed with different dilutions of HER-2. The results show that HER-2 significantly inhibited trastuzumab binding to GST-H98 (Fig. 3*B*). In Fig. 3*B*, GST alone also demonstrated a concentration-dependent inhibition on trastuzumab binding to GST-H98, but the GST-H98 fusion protein doubled or tripled the inhibition effect, as compared with GST. The results demonstrated that peptide H98 played a critical role in inhibiting trastuzumab binding to GST-H98.

Peptide H98 Blocked the Function of Trastuzumab on Inhibiting Cell Growth—Trastuzumab is well known to inhibit the growth of breast cancer cells through interaction with HER-2. Because peptide H98 has the ability to block interaction between trastuzumab and HER-2, we therefore decided to test whether peptide H98 affects the ability of trastuzumab to inhibit cell growth. As shown in Fig. 4, the response of breast cancer cell SKBR3 to trastuzumab was significantly blocked by peptide H98 in a dose-dependent manner and the 50% block rate was reached when peptide H98 was present at a concentration of 60 μg/ml, whereas an equivalent concentration of the control peptide had no significant effect on cell growth inhibition.

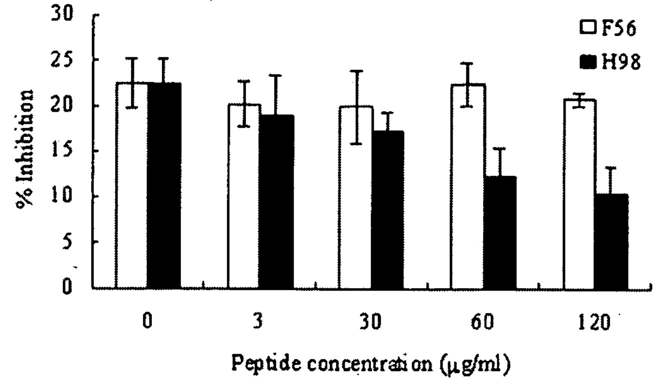

FIG. 4. **Cell proliferation by MTT assay.** SKBR3 cells were plated in 96-well microtiter plates at a density of 1×10^4 cells/well. After incubation at 37 °C overnight, medium containing trastuzumab or human normal IgG (1 μg/ml) with or without peptide at the indicated concentrations was added to the wells. The plates were incubated at 37 °C for 3 days. The numbers of live cells were measured with MTT by reading A_{492}. The proliferation inhibition rate was calculated using the formula $(A_{\text{Human normal IgG}} - A_{\text{Trastuzumab+H98/F56}})/A_{\text{Human normal IgG}} \times 100\%$. S.D. are indicated by *error bars*.

Mice Immunized with GST-H98 Made Anti-HER-2 Antibodies—Peptide H98 was expressed as a GST fusion protein (GST-H98), and the immunogenicities were evaluated in mice. Four of 5 mice immunized with GST-H98 generated peptide-specific antibody (Fig. 5*A*) as well as antibody to HER-2 (Fig. 5*B*) after the fifth immunization. The anti-HER-2 response was lower than the anti-peptide response, and the anti-peptide H98 response could be detected earlier than the anti-HER-2 response. No antipeptide or anti-HER-2 response was observed in mice immunized with GST. One of 5 mice generated antibody to HER-2 that could be detected in an immunoprecipitation Western blot assay (data not shown).

Development of Cellular Immune Response—Cellular immunity induced in mice that were immunized with GST-H98 or GST-other peptide was evaluated with T-cell proliferation assay. The spleens of two mice were isolated for use in this assay. T-cell proliferation was assayed as described under "Experimental Procedures" using H98 as a stimulant. The results shown in Fig. 6 indicate that T-cell proliferation took place in mice immunized with GST-H98 but not in mice immunized with GST-other peptide.

Structural Determinants of H98 Binding to Trastuzumab—Decrease in peptide length may simplify the modifications of the peptide sequence, as well as provide an indication of the characteristics of the binding cleft. The ELISA and Western blot results showed that among 15 kinds of GST-H98 including GST-truncate and GST-mutant peptide fusion protein, only GST-H98, GST-C10, GST-MutN1-2, GST-MutN1, and GST-cyclic H98 were able to specifically bind to trastuzumab (Figs. 7 and 8).

As shown in Figs. 7 and 8, GST-MutN3-4 (mutant of N-terminal GP to VA) and GST-C8 could not bind to trastuzumab, but GST-C10 could bind to trastuzumab. These differences in binding abilities imply that the third and fourth amino acids,

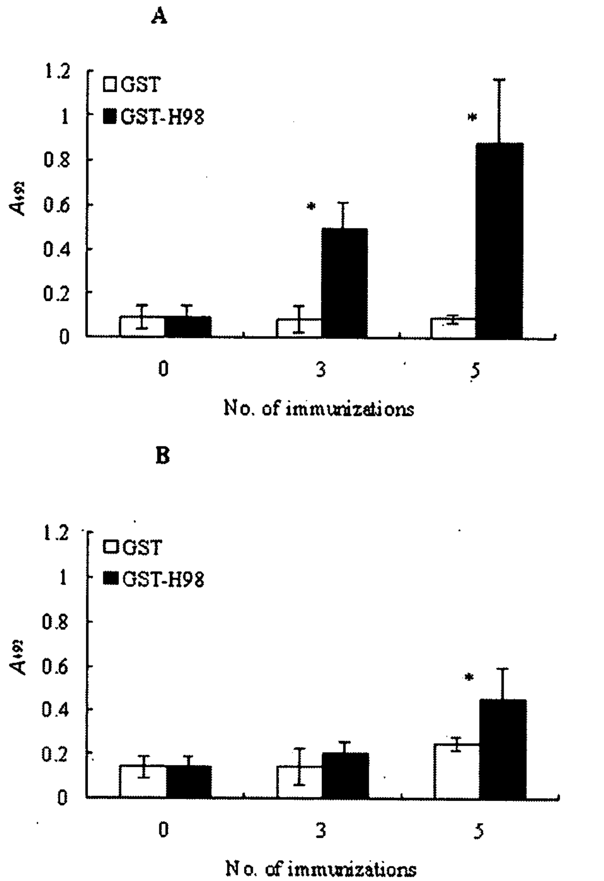

FIG. 5. **Antibody response of mice immunized with GST-H98 or GST.** Sera of mice were collected at different times and tested by ELISA for reactivity against peptide H98 and HER-2. *A* shows the anti-peptide H98 response. Microtiter plates were coated with 5 μg/ml DHFR-H98 fusion protein and then blocked with 5% skim milk. The antisera was diluted 1:1000 and added to the wells. Bound antibody was detected with HRP-conjugated anti-mouse IgG antibody. The values are A_{492} and the average antibody concentrations of five mice. S.D. are indicated by *error bars*. *B* shows the anti-HER-2 response. Microtiter plates were coated with 1×10^4 cells/well of NIH3T3-ErbB2 and then blocked with 5% skim milk. 1:100 diluted mice sera were added to the wells. Bound antibody was detected with HRP-conjugated anti-mouse IgG antibody. The values are A_{492} and the average antibody concentrations of five mice. S.D. are indicated by *error bars*. *, $p < 0.05$ (compared with GST immunized mice, using Student's *t* test).

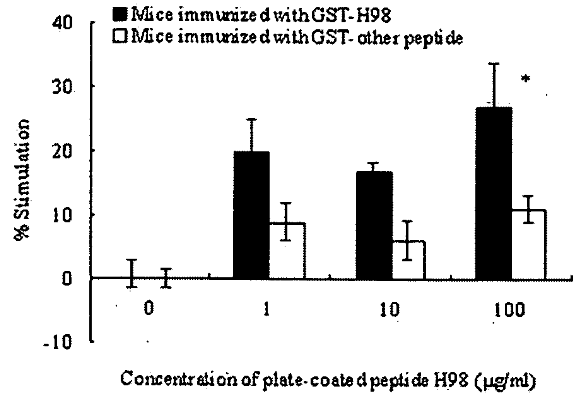

FIG. 6. **T-cell proliferation analysis by MTT assay.** Cellular immune response induced in mice immunized with GST-H98 or GST-other peptide was evaluated with T-cell proliferation assay. T-cell proliferation was carried out as described under "Experimental Procedures" using H98 as a stimulant. The proliferation rate was calculated according to the following formula: $(A_{\mathrm{H98}} - A_{\mathrm{untreated}})/A_{\mathrm{untreated}} \times 100\%$. Each assay was performed at least in triplicate. S.D. are indicated by *error bars*. *, $p < 0.05$ (compared with mice immunized with GST-other peptide, using Student's *t* test).

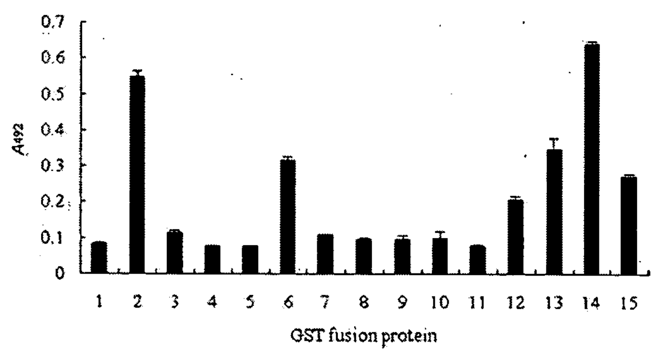

FIG. 7. **Binding of trastuzumab to different GST peptide fusion proteins.** Microtiter wells were coated with 5 μg/ml GST peptide fusion protein and then blocked with 5% skim milk. Two μg/ml trastuzumab was added to the wells. Bound trastuzumab was detected with horseradish peroxidase-conjugated anti-human IgG antibody. The values are A_{492} and the mean of triplicate samples. The *error bars* indicate S.D. *Column 1*, GST; *column 2*, GST-H98; *column 3*, GST-N10; *column 4*, GST-N8; *column 5*, GST-N6; *column 6*, GST-C10; *column 7*, GST-C8; *column 8*, GST-C6; *column 9*, GST-M8; *column 10*, GST-M6; *column 11*, GST-MutN3–4; *column 12*, GST-MutC1–2; *column 13*, GST-MutN1–2; *column 14*, GST-MutN1; *column 15*, GST-cyclic H98.

glycine (Gly) and Proline (Pro), at the N terminus were essential for the activity of peptide H98. Moreover, neither GST-MutC1–2 (mutant of C-terminal SH to GA) nor GST-N10 could bind to trastuzumab, which suggested that serine (Ser) and histidine (His) at the C terminus were also essential to retain complete peptide binding activity. Alternatively, the ability of GST-MutN1–2 to bind to trastuzumab was significantly impaired but not completely abolished, which implied that the first two leucines (LL) at the N terminus were not very important for the binding activity of H98 but were likely to have an effect on the marginal structure formation of the peptide. Further deletion of 4 or 6 residues at the N or C terminus completely abolished the ability of peptide H98 to bind to trastuzumab. Thus it appears that the peptide must be more than 8 amino acids in length to fulfill the requirements of binding.

According to the structure analysis of trastuzumab binding to HER-2 or peptide H98 with the Docking program in the Insight II (2000) software package, the first amino acid of H98 at the N terminus was mutated from leucine (Leu) to glutamine (Gln), which is similar to HER-2[602]Gln and more likely to form hydrogen bonds with trastuzumab (Fig. 9). The binding analysis verified that the interaction between this variant of H98 and trastuzumab was increased (Figs. 7 and 8, *lane 14*). Finally, to test the effect of cyclization on peptide recognition and binding, we introduced two cysteines (Cys) flanking the peptide at the N and C terminus, and found that the affinity of cyclic peptide to trastuzumab is significantly impaired rather than improved (Figs. 7 and 8, *lane 15*).

DISCUSSION

Her-2/neu (c-erbB2), as a 185-kDa glycoprotein ($\mathrm{p185}^{\mathrm{erbB2}}$), is a member of the epidermal growth factor receptor family. The protein $\mathrm{p185}^{\mathrm{erbB2}}$ consists of three domains: a glycosylated extracellular domain with two cysteine-rich regions, a hydrophobic transmembrane domain, and an intracellular tyrosine kinase domain (18). HER-2 overexpression is directly associated with the malignant transformation of epithelial cells. The critical role of HER-2 in epithelial oncogenesis as well as its selective overexpression on malignant tissues makes it an ideal target for cancer biotherapy.

Humoral and cellular immunoreaction to HER-2 are known to occur naturally in patients with HER-2 positive tumors (7, 20, 21). In clinic trials, HER-2 vaccines were restricted to T-cell epitope peptides, which limited the use on some patients because of their major histocompatibility complex haplotype. So the use of B-cell epitope peptide vaccines may complement immunotherapy methods, such as using whole cells, glycosylated extracellular domain, DNA, or T-cell peptide epitopes. However, few studies have described the induction of epitope-specific B-cell immune responses to HER-2. Dakappagari *et al.* (11, 22) identified the B-cell epitopes from HER-2 by computer-aided analysis, and used this epitope to synthesize chimeras with a T-cell epitope from measles virus fusion protein. The chimeric peptide vaccines induced high levels of antibodies,

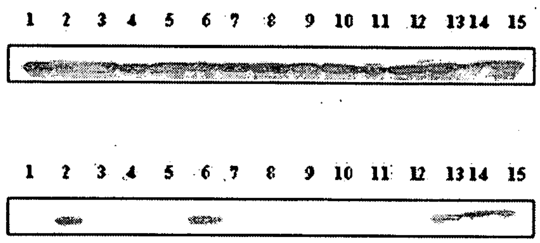

FIG. 8. **Western blot analysis of trastuzumab binding to different GST peptide fusion proteins.** The GST fusion proteins were prepared as described under "Experimental Procedures." Equal amounts (2 μg) of proteins were subjected to SDS-PAGE and transferred to nitrocellulose membranes. Anti-GST antibody (*upper panel*) or trastuzumab (*lower panel*) was used separately as the first antibodies, and an HRP-conjugated secondary antibody was applied to detect the protein reactions as described under "Experimental Procedures." *Lane 1*, GST; *lane 2*, GST-H98; *lane 3*, GST-N10; *lane 4*, GST-N8; *lane 5*, GST-N6; *lane 6*, GST-C10; *lane 7*, GST-C8; *lane 8*, GST-C6; *lane 9*, GST-M8; *lane 10*, GST-M6; *lane 11*, GST-MutN3–4; *lane 12*, GST-MutC1–2; *lane 13*, GST-MutN1–2; *lane 14*, GST-MutN1; *lane 15*, GST-cyclic H98.

which inhibited tumor cell growth, in outbred rabbits (11, 22). Although computer-aided analysis is one method available for finding epitopes, it does not always work well because the immunogenicity of tumor-associated antigen could be affected by glycosylation, which is difficult for a computer to predict. Knowing the crystal structure of trastuzumab binding with human HER-2, we synthesized a epitope peptide (PQCADPPF-GDQ) containing vital amino acids for HER-2 binding with trastuzumab, but this peptide did not show any binding activity with trastuzumab in our experiments (data not shown).

Yip *et al.* (23) used the HER-2 antibody N21 as a target for screening a phage display library of HER-2 gene fragments, and isolated a peptide containing 55 amino acids, which elicited the active immune response to HER-2 in mice. Generally, the epitope can be a linear fragment or conformational region. Linear epitopes correspond to the ordered sequence of amino acids in the protein, whereas in conformational epitopes (mimotope), sequence from non-sequential protein regions in the primary structure or its associated carbohydrates contributes to a single tridimensional site. In view of the fact that phage-displayed HER-2 gene fragment libraries only select linear epitopes, screening phage display peptide libraries may be a simple and convenient way to find out not only linear epitopes but also conformational epitopes. To search for the peptides that could mimic the epitope of HER-2 and would have potential as a vaccine in immunotherapy, Riemer *et al.* (24) used trastuzumab as the target to screen the 10-mer phage display peptide library, and they obtained five candidate mimotopes that could induce an antibody response to HER-2 (24). Alternatively, we screened a 12-mer phage display peptide library by bio-panning also with the antibody trastuzumab as the target protein, and obtained a completely different peptide (LLGPYELWELSH) from Riemer's (C-QMWAPQWGPD-C, C-KLYWADGEFT-C, etc.). In our procedure of screening the library, some improvements were introduced. First, to increase

A B

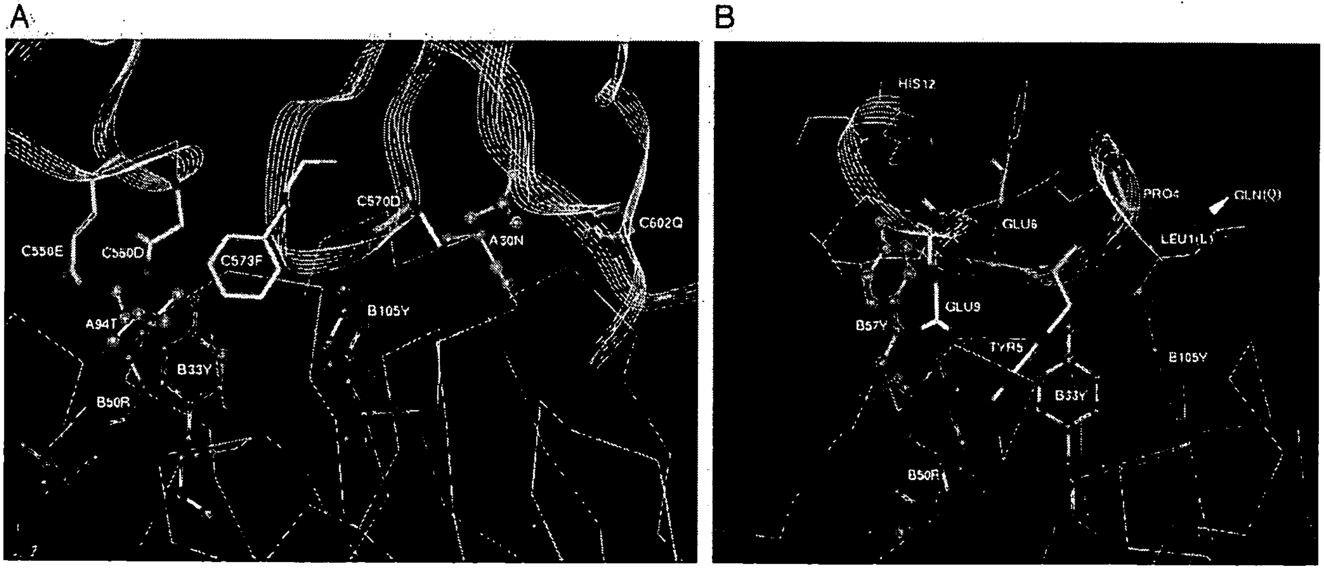

FIG. 9. **Structure analysis of trastuzumab binding to HER-2 or peptide H98 by the Insight II (2000) software package.** *A*, a side view of the HER-2 and trastuzumab binding site. The letters "A," "B," and "C" represent amino acids from trastuzumab κ light chain, heavy chain, and HER-2, respectively. The HER-2 backbone is shown with *green ribbon*. The amino acids involved in the interaction of HER-2 and trastuzumab are labeled in *white*. The trastuzumab chain A (κ light chain) is shown as a *blue line*, whereas chain B (heavy chain) is shown as a *purple line*. The residues HER-2^{560}Asp (shown as "C560D") juxtaposed with HER-2^{573}Phe (shown as "C573F") and HER-2^{572}Pro are in the first and second loop regions of the HER-2 binding site to trastuzumab, based on the published crystal structure 1N8Z (19). *B*, a model of peptide H98 and trastuzumab binding site. The letter "B" represents amino acids from trastuzumab heavy chain. The peptide H98 backbone is shown with the *green ribbon* and amino acids H98-^{6}Glu, H98-^{9}Glu, and H98-^{5}Tyr, which reacted with trastuzumab, are marked in *white*. The interface of trastuzumab chain A (κ light chain) is shown as a *blue line*, whereas the chain B (heavy chain) is shown as a *purple line*. Residues B-^{33}Tyr (shown as "B33Y") and B-^{105}Tyr (shown as "B105Y") in chain B (heavy chain) have π-π-conjugated interaction with H98-^{5}Tyr. In addition, H98-^{6}Glu and H98-^{9}Glu have hydrogen-bond interactions with trastuzumab. The *arrow* indicates the N-terminal mutation from leucine (Leu) to glutamine (Gln).

the contact between the phage-displayed peptides and the antibodies, we used solution bio-panning instead of the solid-phase bio-panning, which is more commonly used. Second, to remove the nonspecific clones, the original phage display library was first pre-absorbed with human serum IgG. Finally, instead of eluting the phages from the target proteins, the elution procedure was omitted in our work by infecting *E. coli* ER2738 directly with phages binding with trastuzumab and protein A complex. Twenty-six positive phage clones were selected from 300 clones and 25 clones were completely identical by DNA sequencing. To rule out the effects of phage protein in the binding, the GST peptide fusion protein was made. The clone GST-H98 showed binding activity to trastuzumab, but GST-H23 did not (data not shown). Further analysis demonstrated that the isolated mimotope H98 not only specifically inhibited the binding of trastuzumab to HER-2, but also blocked the function of trastuzumab in inhibiting the proliferation of cancer cells. Moreover, immunization analysis demonstrated that H98 could induce an active immune response to HER-2 *in vivo*. These results strongly suggested that H98 is similar to the antigen epitope of HER-2 that is recognized by trastuzumab.

Although no sequence homology was found between the mimotope (H98) and HER-2 by sequence alignment, our results appear that H98 mimics a conformational structure of the HER-2. To analyze the binding structure of peptide H98 with trastuzumab, we used the Insight II (2000) software package and a crystal structure of human HER-2 binding with trastuzumab at 2.5 Å (19). Docking program in Insight II (2000) was carried out to model the complex of H98 and trastuzumab. The interaction between HER-2 and trastuzumab buries 1,350 $Å^2$ of surface area over a long groove and possesses an unusually high shape complementarity for antigen-antibody interactions. The interaction was mediated by three regions of HER-2: loops formed by residues 557–561 (PEADQ), 570–573 (DPPF), and the base of a loop formed by residues 593–603 (KFPDEEG-ACQP). Interactions formed by the first and third loop were primarily electrostatic, whereas the second loop made mostly hydrophobic contacts in a pocket formed by the CDR3 loops of heavy and κ light chains of the antibody. The crystal structure of HER-2 with trastuzumab showed that HER-2^{560}Asp is surface exposed and in juxtaposition to HER-2^{573}Phe and HER-2^{572}Pro, which are also surface exposed. These three amino acids, HER-2^{560}Asp-HER-2^{573}Phe-HER2-^{572}Pro, form an epitope of HER-2 in structure and were well represented by the sequence "H98-^{6}Glu-H98-^{5}Tyr-H98-^{4}Pro" in peptide H98. Amino acids HER-2^{560}Asp and H98-^{6}Glu are similar in structure, as are HER-2^{573}Phe and H98-^{5}Tyr. Moreover, HER-2^{558}Glu could be replaced by H98-^{9}Glu to form hydrogen bonds with heavy and κ light chains of trastuzumab (Fig. 9). This epitope structure analysis demonstrated that H98 could mimic the epitope of HER-2 binding to trastuzumab in structure, which is consistent with our experimental results. Moreover, knowing that HER-2^{602}Gln could form hydrogen bonds with trastuzumab, we made a mutant (MutN1) in which the first amino acid of H98 was changed from leucine (Leu) to glutamine (Gln) (Fig. 9*B*). The mutant H98 achieved a higher binding activity to trastuzumab. To identify the critical amino acids in H98 for its binding to trastuzumab, we made truncation and mutation variants of the peptide H98 (see Table I). The binding assays showed that the last two amino acids at the C terminus and the third together with the fourth amino acid at the N terminus play an important role in the binding of H98 to trastuzumab.

In summary, peptide H98, which was isolated from the phage display peptide library with trastuzumab, could mimic the antigen epitope of HER-2 on a conformation structure and successfully elicit the humoral and cellular immune responses to HER-2. This peptide shows promise for development as a HER-2 vaccine for biotherapy of cancer that overexpresses HER-2.

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Cell Biology

Mechanistic aspects of the opposing effects of monoclonal antibodies to the ERBB2 receptor on tumor growth

(growth factors/tyrosine kinase/adenocarcinoma/oncogene)

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Contributed by Michael Sela, July 11, 1991

ABSTRACT **The *ERBB2* (also called *HER2*, *neu*, and *c-erbB-2*) gene product, which encodes a growth factor receptor, was implicated in the malignancy of human adenocarcinomas. An antibody directed to the rat oncogenic receptor has been previously shown to have an antitumor effect in model systems. In an attempt to extend this observation to the protooncogenic human receptor and also to understand the underlying mechanism, we generated a panel of monoclonal antibodies specific to the extracellular portion of the ERBB2 protein. The effects of the antibodies on tumor growth were compared with their cellular and biochemical actions *in vitro*. Surprisingly, opposing *in vivo* effects were observed: although some antibodies almost completely inhibited the growth in athymic mice of transfected murine fibroblasts that overexpress Erbb-2, other antibodies either accelerated tumor growth or resulted in intermediate responses. When tested on cultured human breast carcinoma cells or ERBB2 transfectants, the tumor-stimulatory antibody was found to induce significant elevation of tyrosine phosphorylation of the ERBB2 protein. In contrast, only partial correlation was observed between the capacity to restrict tumor growth and the effects of the antibodies on receptor degradation and cellular proliferation *in vitro*. This suggests that the antitumor antibodies affect both receptor function and host–tumor interactions. Our results may help establish experimental criteria for the selection of specific antibodies for use either alone or in conjunction with other molecules as pharmacological antitumor agents.**

Evidence has been accumulated in recent years for the involvement of growth factors and their receptors in the process of malignant transformation. The ERBB2 protein is a receptor tyrosine kinase (1), homologous to the epidermal growth factor (EGF) receptor (2, 3). The rat homologue of the gene undergoes oncogenic activation through a single point mutation (4). The ERBB2 protein was found to be overexpressed in several types of human adenocarcinomas, especially in tumors of the breast and the ovary (5–7), and the overexpression was correlated with short time to relapse and poor survival of breast cancer patients (5).

The potential use of monoclonal antibodies (mAbs) in diagnosis and treatment of cancer has been studied extensively (8). Receptors for growth factors constitute an ideal target for this approach because their location on the cell membrane makes them accessible to antibody molecules. Moreover, antibodies directed to growth factor receptors can potentially block biological functions essential for cell proliferation. Previous studies have demonstrated, in model systems, the potential therapeutic effect of mAbs against the epidermal growth factor receptor (9, 10). Likewise, different mAbs to the ERBB2 receptor inhibited the proliferation of a human breast carcinoma cell line in culture (11), and an antibody directed to the rat ERBB2 protein inhibited the tumorigenicity of fibroblasts transformed by the mutated rat *ERBB2* oncogene (12, 13). mAbs that recognize the protein product of the human ERBB2 protooncogene have been raised and used to study the biological function of the presumed receptor (14–16).

Our studies were aimed at the generation of antibodies with potential use in immunotherapy of human cancer, either alone or conjugated with drugs or toxins. To this end we raised a panel of mAbs to the extracellular portion of the ERBB2 receptor. These antibodies induced opposing effects on tumor growth in athymic mice. Our attempts to analyze the mechanism of antibody-mediated tumor enlargement suggest that activation of the tyrosine kinase is involved, but inhibition of tumor growth is not simply correlated with one receptor function.

MATERIALS AND METHODS

Chemicals and Reagents. Affinity-purified goat anti-mouse $F(ab')_2$ was from Jackson ImmunoResearch. It was radiolabeled with $Na^{125}I$ (Amersham) by the chloramine T procedure (17). $[^{32}P]$Orthophosphate was from the Nuclear Research Center (Negev, Israel); $[^{35}S]$methionine and $[\gamma\text{-}^{32}P]$ATP were from Amersham. Sepharose-protein A was purchased from Pharmacia. The anti-phosphotyrosine mAb 1G2 (18) was purified from ascites fluid.

Cell Lines. The HER2 cell line has been described (19). The SKBR3 human breast carcinoma cell line was obtained from the American Type Culture Collection.

Experimental Animals. BALB/c mice, CB6/F_1 mice, and CD1/nude mice were obtained from the Experimental Animals Center of the Weizmann Institute of Science.

Generation of mAbs to the ERBB2 Receptor. BALB/c mice (2 mo old) were injected i.p. three times with 3–5 × 10^6 living SKBR3 human breast carcinoma cells, at intervals of 2 weeks. Antisera were tested by an immunoprecipitation assay using HER2 cells (NIH 3T3 cells transfected with human *ERBB2* gene, ref. 19), labeled metabolically with $[^{35}S]$methionine. The spleens of mice that developed a strong immune response were selected for fusion. The spleen cells were fused with NSO myeloma cells by using polyethylene glycol (20), and the hybridomas were selected with hypoxanthine/aminopterin/thymidine medium. Supernatants of the growing cells were screened by using an indirect binding assay. CHO cells transfected with the *ERBB2* gene (HCC cell line) were plated on a flexible 94-well plate, previously coated with polylysine (1 mg/ml). The cells were fixed with 3% (wt/vol) paraformaldehyde, and supernatants of hybridomas were incubated for 1 hr at 22°C with the fixed cells. The bound

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Abbreviation: mAb, monoclonal antibody.

antibodies were detected with ^{125}I-labeled goat anti-mouse $F(ab')_2$ antibody. As a negative control we used the parental, untransfected CHO cell line.

The antibodies that specifically bound to the HCC cells were selected for further analysis by using either an immunoprecipitation assay with [^{35}S]methionine-labeled cells or immunoprecipitation followed by autophosphorylation in the presence of $MnCl_2$ and [γ-^{32}P]ATP (21). Positive hybridomas were cloned twice by limiting dilution. Determination of antibody class was done with class-specific second antibodies. Large quantities of specific mAbs were produced by preparation of ascites fluid in CB6/F_1 mice. The IgM antibody was separated on a Sephacryl G300 column, and the IgG1 and IgG2a antibodies were purified by affinity chromatography on Sepharose-protein A, using elution conditions specific for each subclass.

Indirect Binding Assay on Living Cells. SKBR3 cells or HER2 cells were plated in 24-well plates and assayed at confluence. The cells were incubated for an hour at 22°C with various concentrations of antibodies in phosphate-buffered saline (PBS)/1% bovine serum albumin. After being washed with the same buffer, the cells were incubated for 90 min with ^{125}I-labeled goat anti-mouse $F(ab')_2$ (10^5 cpm per well). The cells were then washed and solubilized with 0.1 M NaOH; the radioactivity was then determined in a γ counter.

Determination of the *in Vivo* Effect of the mAbs. HER2 cells (3×10^6) were injected s.c. into nude mice, followed by three i.p. injections of the mAbs on days 3, 7, and 10. Tumor parameters were measured twice a week with callipers, and tumor volume was calculated according to the formula: tumor volume equals length × width × height. To validate volume measurements the correlation between the tumor volume and tumor weight was determined on the day of animal killing.

Determination of Tyrosine Phosphorylation in Living Cells. The SKBR3 or HER2 cells were grown in a 24-well plate and labeled for 4 hr in Dulbecco's modified Eagle medium (DMEM) without phosphate but in the presence of 1% dialyzed fetal calf serum and [^{32}P]orthophosphate (0.5 mCi/ml; 1 Ci = 37 GBq). The cells were washed with PBS and incubated for 15 min at 22°C with fresh medium containing antibodies at a concentration of 10 μg/ml. After being washed, the cells were lysed in solubilization buffer (21), and the tyrosine-phosphorylated ERBB2 protein was immunoprecipitated with an agarose-immobilized antibody to phosphotyrosine (18). The immune complexes were eluted with solubilization buffer containing 50 mM *p*-nitrophenylphosphate and subjected to immunoprecipitation with a rabbit polyclonal anti-ERBB2 antibody, directed to the carboxyl terminus of the receptor (21).

Determination of the Effect of the mAbs on Receptor Turnover. SKBR3 or HER2 cells were grown in 24-well plates to 80% confluence and then labeled for 16 hr at 37°C with [^{35}S]methionine (50 μCi/ml). After being washed with PBS, the cells were incubated with fresh medium in the absence or presence of the antibodies (at a concentration of 10 μg/ml) for various periods of time. The cells were then washed, and cell lysates were subjected to immunoprecipitation with a rabbit polyclonal antibody to the ERBB2 protein (21).

Complement-Dependent Cytotoxicity (CDC) Assay. The SKBR3 tumor cells were incubated at 37°C for 2 hr in a volume of 0.1 ml of fetal calf serum, with 300 μCi of Na[^{51}Cr]O_4 (DuPont/NEN). At the end of the labeling period the cells were washed three times in PBS, and 1.5×10^4 cells (in 25 μl) were plated in each well of a 96-well microtiter plate. Various concentrations of the mAbs (25 μl) were added and incubated with the cells for 1 hr followed by the addition of human or rabbit complement and incubation for further 3 hr. Appropriate control wells containing cells alone, cells with no antibody or no complement, and cells lysed in 10% SDS were set up in parallel. ^{51}Cr release was determined in a γ counter. The means of triplicate determinations are given.

Antibody-Mediated Cell-Dependent Cytotoxicity (ADCC) Assay. The SKBR3 tumor cells were labeled with Na[^{51}Cr]O_4 as described above. Cells (5×10^3) in 25 μl were incubated for 1 hr with various concentrations of the mAbs and then for 5 hr with effector cells, human peripheral blood lymphocytes (0.1 ml, lymphocytes/tumor cells = 140:1), or with mouse splenocytes (120:1). ^{51}Cr release was determined as described above.

RESULTS

Generation of mAbs Directed to the ERBB2 Receptor. Five hybridomas were selected after the fusion of NSO myeloma cells with splenocytes obtained from mice immunized with intact cells of the human breast carcinoma SKBR3 cell line. This immunization procedure elicited specific antibodies to the extracellular portion of the human ERBB2 antigen. The isotypes and subclasses of the resulting mAbs are given in Table 1. Three of these antibodies were found to be of the IgG1 subclass (N12, N24, N28), one was an IgM (N10), and one an IgG2a antibody (N29). As depicted in Fig. 1, all the mAbs specifically bound to cultured cells that express the ERBB2 receptor, yet they bound with different apparent affinities. Antibodies N28 and N24 displayed the highest apparent affinity, whereas N10 mAb exhibited the lowest apparent affinity. All five mAbs immunoprecipitated a single protein of 185 kDa from metabolically labeled HER2 cells, as shown in Fig. 2*A*. This result was also reflected in an *in vitro* kinase assay performed on the immunoprecipitates (Fig. 2*B*). None of these mAbs reacted with the epidermal growth factor receptor or with the rat p185^{neu} (data not shown). Immunoblot analysis of the ERBB2 protein showed that only the N12 and N29 antibodies could react with the denatured form of the receptor (Table 1).

The Effect of mAbs upon Tumor Growth *in Vivo*. The mAbs were assayed for their ability to affect tumor growth of murine fibroblasts transformed by overexpression of the *ERBB2* gene (HER2 cells), in nude mice. The mAbs or a control antibody to dinitrophenol (anti-DNP) were injected i.p., into groups of five mice, on days 3, 7, and 10 after tumor inoculation. Fig. 3*A* depicts tumor volumes of each group of mice, on day 21, postinoculation. The tumorigenic growth of HER2 cells was significantly inhibited ($P < 0.05$, as calculated by using Duncan's multiple comparison test) in nude mice injected with mAbs N29 and N12, when compared with mice that received no antibody or the control anti-dinitrophenyl antibody. As depicted in Fig. 3*B*, the inhibitory

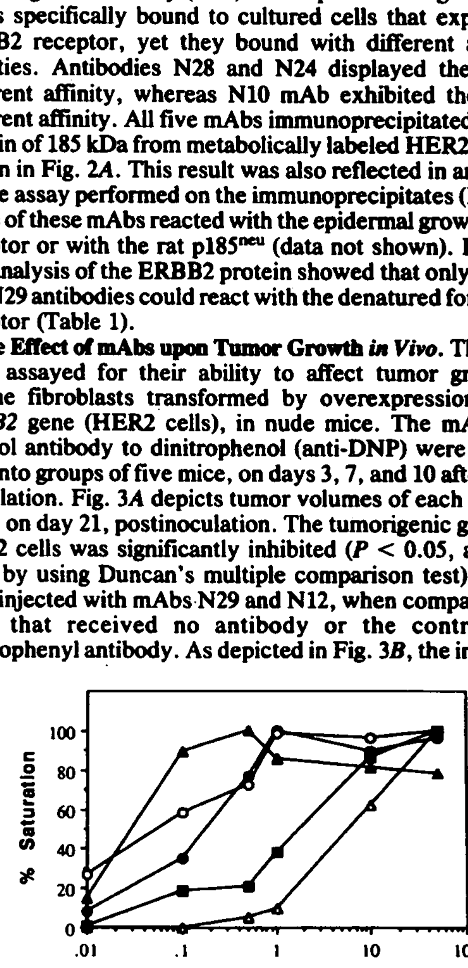

FIG. 1. Binding of monoclonal anti-ERBB2 antibodies to HER2 cells. Confluent monolayers of HER2 cells were incubated for 1 hr at 22°C with various concentrations of mAbs. Bound antibodies were subsequently determined with ^{125}I-labeled goat anti-mouse $F(ab')_2$. Control cells were incubated without the murine antibody, and their background binding was subtracted. △, N10 (IgM); ●, N12 (IgG1); ○, N24 (IgG1); ▲, N28 (IgG1); ■, N29 (IgG2a).

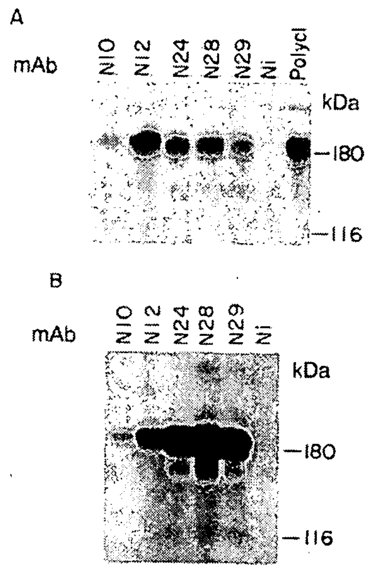

FIG. 2. Immunoprecipitation of the ERBB2 protein by mAbs. (*A*) HER2 cells were metabolically labeled with [^{35}S]methionine, and the cell lysates were separately subjected to an immunoprecipitation assay with 10 μg of each mAb. As a control nonimmune serum (Ni) was used. Proteins were separated on a SDS/7.5% polyacrylamide gel. (*B*) The immunoprecipitation assay was done as described but with unlabeled cells. Before electrophoresis, the proteins from the cell lysate were labeled by autophosphorylation with [γ-^{32}P]ATP and 10 mM $MnCl_2$. Autoradiograms are shown. NI, nonimmune serum; Polycl, polyclonal anti-ERBB2 antibody.

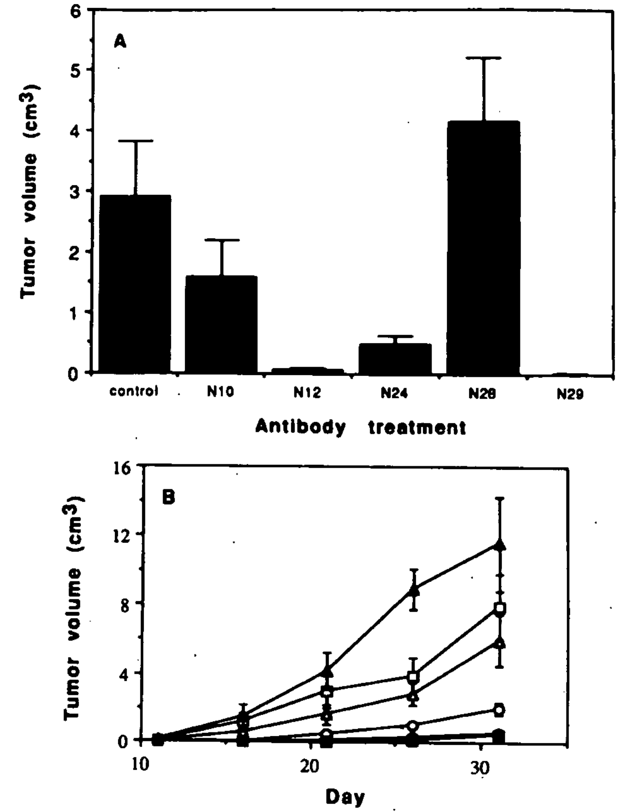

FIG. 3. Effect of mAbs on tumor growth in athymic mice. Cells (3×10^6) were injected, s.c. into CD1/nude mice. Groups of five mice received three i.p. injections on days 3, 7, and 10 at a total mAb dose of 2 mg per mouse. Tumor size was measured as described. As control, an irrelevant antibody anti-dinitrophenol or buffer alone (PBS) was used. (*A*) Effects of antibody treatment after 21 days postinoculation. (*B*) Kinetics of tumor growth in antibody treated mice. □, Control; △, N10; ●, N12; ○, N24; ▲, N28; and ■, N29. Statistical analysis was done by using the analysis of variance and Duncan's multiple comparison test.

effect persisted over 31 days after tumor injection. Antibodies N10 and N24 exhibited less efficient inhibition of tumor growth. In contrast, mAb N28 consistently stimulated tumor growth. Essentially identical results were obtained in three separate experiments. To test the possibility that the effects seen *in vivo* are reflected *in vitro*, we used cell proliferation assay in culture and cytotoxicity assays on SKBR3 human breast tumor cells. Partial, if any, correlation was found between the results obtained in these assays and the *in vivo* experiments (Table 1).

Stimulation of Tyrosine Phosphorylation of ERBB2. It has been shown (22) that mAbs directed against the rat ERBB2 protein elevated tyrosine phosphorylation of this receptor. Two different assays were used to test the capacity of our mAbs to elevate tyrosine phosphorylation of the ERBB2 protein: HER2 cells were metabolically labeled with [^{32}P]orthophosphate, incubated with the mAbs, and subjected to two consecutive immunoprecipitation steps with anti-phosphotyrosine and anti-ERBB2 antibodies (21). Alternatively, SKBR3 cells were first incubated with the mAbs and then subjected to two consecutive immunoprecipitation steps, followed by an *in vitro* phosphorylation assay in the presence of [γ-^{32}P]ATP and $MnCl_2$. As depicted in Fig. 4, similar results were obtained in both experiments: mAb N28 significantly stimulated phosphorylation of the ERBB2 receptor on tyrosine residues, whereas the other mAbs displayed low (N12, N24, N29 mAbs) or no activity (N10 antibody) in living cells.

The Effects of the mAbs on the Rate of Receptor Turnover. The interaction of receptor tyrosine kinases with their respective ligands is usually coupled to rapid endocytosis. It was further shown that antibodies could induce an analogous effect on the rat ERBB2 receptor (22) and that this activity was associated with disappearance of the transformed phenotype (23). We, therefore, tested the potential of our mAbs to the human ERBB2 protein to accelerate the turnover of the receptor. For this purpose, HER2 cells were biosynthetically labeled with radioactive methionine and then chased for various periods of time with fresh medium that contained different mAbs. At the end of the chase period, the residual labeled proteins were immunoprecipitated and analyzed by gel electrophoresis and autoradiography. The results of this experiment are shown in Fig. 5: all the mAbs accelerated, to different extents, the rate of turnover of the receptor, with antibody N29 being the most effective.

DISCUSSION

Overexpression of ERBB2 protein is frequently found in human adenocarcinomas, and it is believed to be involved in the progression of the malignancy state (5–7). This possibility was supported by gene transfer experiments demonstrating that overexpression of the apparently normal gene, driven by heterologous promoters, confers tumorigenicity on murine fibroblasts (19, 24). These observations, together with the tyrosine kinase activity of the ERBB2 protein, have made this human receptor an excellent target candidate for antibody-mediated therapy of human solid tumors. Indeed, many different mAbs to the human protein have been generated (14–16), but their anti-tumor activity was not extensively investigated *in vivo*. On the other hand, a mAb to the rat ERBB2 protein efficiently inhibited the growth of tumori-

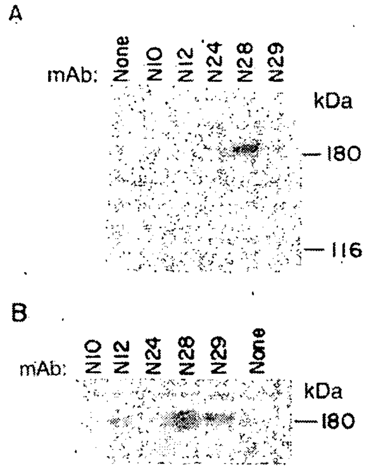

FIG. 4. Antibody-induced stimulation of tyrosine phosphorylation of the ERBB2 receptor. (*A*) Monolayers of HER2 cells were labeled with [^{32}P]orthophosphate and then incubated for 15 min at 22°C with each antibody at 10 μg/ml. Tyrosine-phosphorylated proteins were immunoprecipitated with an anti-phosphotyrosine antibody, followed by specific elution and a second immunoprecipitation step with rabbit anti-ERBB2 polyclonal antibody, directed to the carboxyl terminus of the protein. (*B*) SKBR3 cells were first incubated with the antibodies, immunoprecipitated in two consecutive steps, as described above, and labeled by autophosphorylation with [γ-^{32}P]ATP and Mn^{2+}. The autoradiograms of the SDS/gel-separated proteins are shown.

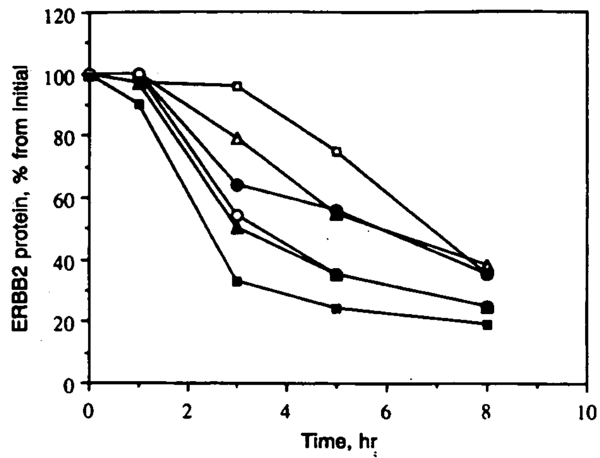

FIG. 5. Effect of mAbs on the rate of turnover of the ERBB2 receptor. HER2 cells were labeled with [^{35}S]methionine in a 24-well plate and then chased for the indicated periods of time with fresh medium that contained the indicated mAbs. Residual ^{35}S-labeled ERBB2 protein was subjected to immunoprecipitation with a rabbit polyclonal antibody directed to the carboxyl terminus of the protein and separated on SDS gel. Quantitative analysis of receptor turnover is shown, as determined by measuring densitometry of the autoradiogram. □, Control cells without antibody treatment; △, N10; ●, N12; ○, N24; ▲, N28; and ■, N29-antibody-treated cells.

genic cells carrying the oncogenic mutated ERBB2 protein (12, 13).

In the present study we used a murine model system to address the potential and diversity of mAbs to ERBB2 as anti-tumor agents. We further attempted to understand the mechanisms of action of the antibodies in the hope that this may constitute an experimental basis for selection of an optimal mAb. Of the five mAbs surveyed in this study, two almost completely inhibited tumor growth, two displayed moderate inhibitory effects, and the last one significantly accelerated the rate of tumor growth (Fig. 3). These differential biological activities can be attributed to different epitopes on the exoplasmic portion of the receptor. The mechanism by which different receptor regions may mediate opposing effects on tumor growth is apparently important for both receptor structure–function relationships and also for the elucidation of the biochemical mechanism underlying tumor inhibition. One simple explanation may be that the ligand-binding site of the putative receptor (25, 26) is involved in the action of the biologically active antibodies. However, in the absence of a well-characterized ligand for the ERBB2 protein this possibility cannot be experimentally tested.

Aware of its limitations, we tried to find a correlation between the *in vivo* effects of the mAbs and their actions on cultured cells. The results of this analysis are summarized in Table 1; in contrast with our inability to correlate the binding

Table 1. Comparison of the biological properties of anti-ERBB2 mAbs

Antibody	Immunoblot*	Tumor growth,† %	Cell proliferation,‡ %	CDC,§ %	ADCC,¶ %	Tyrosine phosphorylation,‖ -fold	Receptor degradation,** $t_{1/2}$ in hr
Anti-DNP	−	100	100	ND	ND	1.0	6.5
N10 IgM	−	54	247	68 ± 3.1	7 ± 1	0.9	6
N12 IgG1	+	2	63	9 ± 0.9	10 ± 0.01	1.8	6
N24 IgG1	−	16	196	60 ± 1.1	9 ± 2.2	2.5	3.5
N28 IgG1	−	141	107	10 ± 1.7	18 ± 0.01	14.0	3
N29 IgG2a	+	0.3	72	9 ± 2.2	12 ± 0.33	1.2	2.5

DNP, dinitrophenol; CDC, complement-dependent cytotoxicity; ADCC, antibody-mediated cell-dependent cytotoxicity, ND, not determined.
*HER2 cell lysates were separated by SDS/gel electrophoresis, transferred to nitrocellulose, and blotted with the mAbs, followed by detection with horseradish peroxidase-conjugated goat anti-mouse F(ab')$_2$.
†Average tumor volume (as percentage of control; n = 5) measured 21 days after tumor inoculation.
‡SKBR3 breast tumor cells were plated in 24-well plates (10^3 cells per well) and incubated for 48 hr in medium supplemented with 10% fetal calf serum. The amount of serum was then decreased to 5%, and the indicated antibodies were added at 10 μg/ml. Five days later, the numbers of viable cells were determined.
§Complement-dependent cytotoxicity assay of SKBR3 tumor cells was done as described. Values represent [^{51}Cr]O_4 release from cells treated with the indicated mAbs (50 μg/ml) and human complement, as percentages of total cellular content of ^{51}Cr. Corrections were made for spontaneous release, in the absence of antibody and complement. Similar results were obtained by using rabbit complement.
¶Antibody-mediated cell dependent lysis of SKBR3 cells was assayed as described, using each antibody at 50 μg/ml, and human effector cells, and expressed as percentages (see §). Similar results were obtained with mouse splenocytes.
‖Extent of induction of tyrosine phosphorylation of ERBB2 protein by mAbs was determined by densitometry of autoradiograms, according to the assay of Fig. 4*A*.
**Down-regulation of ERBB2 protein was determined with [^{35}S]methionine-labeled HER2 cells, as described in text and Fig. 5, and expressed as half-life of the labeled protein ($t_{1/2}$).

affinities of the various antibodies (Fig. 1) or their effects on cell proliferation in culture with their actions on tumors, an interesting correlation was found with direct responses exhibited by the receptor protein. Thus, our single tumor stimulatory mAb, N28, dramatically stimulated the tyrosine kinase activity of the receptor (Fig. 4). On the other hand, both tumor-inhibitory antibodies were the only mAbs capable of recognizing the fully denatured protein. This may reflect similar characteristics of the epitopes recognized by these antibodies, but the correlation to tumor effect is not readily apparent. Although the most efficient tumor inhibitory antibody, N29, led to the shortest receptor half-life (Fig. 5), this correlation is difficult due to the effects seen with the other antibodies.

The simplest interpretation of these observations is that the positive effect on tumor growth involves stimulation of the tyrosine kinase function of the ERBB2 receptor, whereas tumor inhibition may involve other effects including reduction in the cellular level of intact receptor-kinase molecules. The *in vivo* and *in vitro* effects of mAbs N28 and N29 are in line with the oncogenic role of the overexpressed ERBB2 protein and are also consistent with the tumor-inhibitory function of a mAb directed to the rat ERBB2-transforming protein (12). Nevertheless, tumor inhibition may occur even without a significant effect on receptor down-regulation, as reflected by the action of the N12 mAb. It is, therefore, conceivable that several independent mechanisms may lead to inhibition of tumor growth. Cellular proliferation of either SKBR3 cells (Table 1) or HER2 cells (data not shown) in the presence of the mAbs turned out to be a limited predictor of the anti-tumor potential of each mAb (Table 1). What is the significance then of the lack of reflection *in vitro* of the effects of the various antibodies on tumors *in vivo*? One possible explanation is that the antibodies interfere with a process that occurs only in the living animal. This process may involve changes in tumor invasiveness, attraction of blood vessels (angiogenesis), or a paracrine loop. Interestingly, the differential tumor inhibitory potential of the mAbs also did not correlate with cell lysis *in vitro* (Table 1), suggesting that neither complement- nor antibody-mediated cell lysis significantly contributes to the inhibitory function.

In summary, our results stress the caution with which antibody therapy should be considered, as different mAbs to the same protooncogenic receptor may have opposing effects on tumor growth. Nevertheless, the presented study provides further support to the notion that overexpression of a growth factor receptor leads to oncogenic transformation. It also demonstrates that a carefully selected mAb may be an efficient antitumor agent, at least in an experimental animal system.

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